ADVANCES IN PROTOPLAST RESEARCH

Symposia Biologica Hungarica 22

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22

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Proceedings of the 5th International Protoplast Symposium

July 9–14, 1979
Szeged, Hungary

Edited by
L. FERENCZY and G. L. FARKAS

Associate Editor
GABRIELLA LÁZÁR

(Symposia Biologica Hungarica 22)

The Symposium, the largest on this topic so far, reflected a rapidly expanding interest of microbiologists, botanists, biochemists, geneticists, biophysicists, and agronomists in protoplast research. Throughout emphasis is put on the need to integrate the experimental results and "know how" of various disciplines to exploit the potentialities of the protoplasts, the naked cells, in the solution of a wide array of problems in basic and applied (industrial and agricultural) research. The optimal conditions of isolation, culture and regeneration of protoplasts, recent findings in intra- and interspecific somatic hybridization as well as in cell organelle transfer are discussed in detail. The physiological, biophysical and biochemical characteristics of the protoplast system and, last but not least, its suitability for genetic transformation in eukaryotes are further topics covered. This extensive compilation of ideas and results will undoubtedly stimulate further research and application of scientific results in this rapidly developing field.

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C O N T E N T S

F O R E W O R D

This volume contains a selection of papers presented at the Fifth International Protoplast Symposium. The meeting, jointly organized by the Department of Microbiology, Attila Jozsef University, and the Institutes of Genetics and Plant Physiology, Biological Research Center, Hungarian Academy of Sciences, was held in Szeged, Hungary, July 9-14, 1979.

The symposium clearly demonstrated a rapidly growing interest in protoplast research. This was reflected not only in the number of the countries represented - our guests came from 33 countries - but also in the number of participants and contributions, exceeding the figures of 300 and 140, respectively.

A wide spectrum of results in bacterial, fungal and plant protoplast research was discussed, the major interest, however, centered around the fusion of protoplasts and the characterization of the fusion products.

The contributions published in this volume will provide the reader with a representative cross-section of protoplast research to-day. We sincerely hope that the Proceedings will stimulate investigations in both basic and applied fields in protoplast research and other areas of modern biology.

The Editors

PROTOPLASTS: PAST AND PRESENT

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The term 'protoplast' has its origin in the cytology of plants and is used in Botany to describe the organized entity of the living components of the plant cell which carries out active metabolism, biosynthesis and energy transfer, in contrast to the extra-cellular, essentially metabolically inactive secondary plant matter. This explains why the protoplast is a unifying biological entity: workers in microbiology, whether they are studying fungi or bacteria, algologists, fern and bryophyte investigators, higher plant investigators and animal biologists are brought together in a study of this basic biological homeostatic unit. Hanstein in 1880 (1) was probably the first to use the term 'protoplast' and to have isolated protoplasts; his drawings showed protoplasts within the cell walls of plants and the release of protoplasts and portions of the protoplasts from cells of *Vaucheria* (for illustrations see (2)). Close parallels between plasmolysis in plant and fungal cells and successful plasmolysis in various bacteria appear very early in the microbiological literature (3). From these studies it was established that a functional semi-permeable plasma-membrane surrounded the protoplast.

Following plasmolysis of cells of leaf tissue of *Stratiotes aloides*, Klercker (4) was the first in 1892, to isolate protoplasts. The method employed was similar to that described later by Chambers and Hofler (5) - a few protoplasts could be isolated by using thin slices of epidermis of onion scale immersed in 1.0 M sucrose until the protoplasts had shrunk away from their enclosing walls, and then cutting sheets of the epidermis with a sharp knife. Understandably this mechanical method of isolation was less readily applicable to algae and fungi, and bacteria presented a formidable problem because of their small size. A significant development was the description by Giaja in 1919 (6) of the isolation of protoplasts from yeast cells by digesting the cell wall by means of gastric juice obtained from the snail *Helix pomatia*. The realisation that enzymatic methods for protoplast isolation might provide a method for the large-scale isolation of protoplasts had to await for its implementation on studies on the enzymatic degradation of isolated cell walls, and on their basic composition.

A major stimulus for this came from the work of Salton in 1952 (7) which showed that isolated cell walls of *Micrococcus lysodeikticus* were dissolved by lysozyme; this led to the use of lysozyme (8,9) for the isolation of protoplasts from *Bacillus megaterium*. Interestingly polyethylene glycol (7.5%) was used to stabilise these protoplasts osmotically (9). (For

details of the various procedures for the conversion of bacteria into protoplasts see (10,11). This early work on bacterial protoplasts was dominated by consideration of the extent to which wall removal had occurred, particularly the problems of isolating protoplasts from Gram-negative bacteria. After disintegration of bacterial protoplasts by osmotic shock, extensive studies were also carried out on the nature and biochemical properties of resulting subcellular fragments, particularly the cytoplasmic membrane. Studies on the interaction of bacteriophages with protoplasts of *B.megaterium* showed them to be resistant to phage, which is infective for the bacillary form of the organism: this established the need for phage receptor sites on the cell surface associated with the presence of the cell wall. It also provided some suggestion that such protoplasts could be readily infected by the DNA of the phage (12). These basic studies on bacterial protoplasts re-stimulated interest in the isolation of protoplasts from yeasts and from filamentous fungi, and particularly the use of methods involving enzymatic wall degradation. Yeast protoplasts were isolated in 1957 by Eddy and Williamson (13) using the basic procedure of Giaja (1919) (6) involving use of enzymes present in the digestive juice of snails - later extended to the use of microbial enzyme preparations (14). A considerable amount of work was devoted to the evaluation of the capability of both bacterial and fungal protoplasts to rebuild rigid cell walls, and to revert to normal viable cells. These studies were particularly important because they established that such protoplasts possessed major synthetic capabilities: they also laid the foundation for the genetic manipulation of protoplasts - methods which were however to post-date these earlier basic studies.

Comparable enzymatic methods for the isolation of plant protoplasts only became available in 1960. At that time although much was known about the composition of plant cell walls, and various cellulases were available commercially, nobody had successfully utilised such cell wall degrading enzymes for the isolation of protoplasts. At that time I tested about twenty different cellulase preparations from commercial sources to see if protoplasts could be isolated from plant roots. All were negative. Knowing that there had been extensive work on cellulose degrading enzymes during the Second World War (principally I am told because of fungal attack on American tents in the Pacific) I obtained a sample of *Myrothecium verrucaria* cellulase from Dr. Whitaker of the Canadian National Research Council early in 1960 - it was sent to me in January 1960, and I still have a small sample of this relatively crude enzyme preparation. In his covering letter Dr. Whitaker informed me that its action on crystalline cellulose would be slow, with the implication that it would be unsuitable for attempts to isolate protoplasts. I therefore put his sample at the bottom of the deep freeze - only when everything else had failed to do anything did I test his preparation - it produced protoplasts! (15), and indeed was the only active enzyme mixture that I had available for several years until the development of commercially available cellulases in Japan for protoplast isolation (16).

Against this general historical background it is understandable that the first International Symposium of Protoplasts should have been one on Yeast Protoplasts. This was held at Jena at the Institut fur Mikrobiologie und Experimentelle Therapie, in September 1965 (17). Since that time a series of International Symposia have been held: the second in Brno in 1968 (Second International Symposium on Yeast Protoplasts) (18): the third in Salamanca in 1972 (Third International Symposium on Yeast Protoplasts) (19): the fourth in Nottingham in 1975 (Fourth International Symposium on Yeast

and Other Protoplasts) (20) and the fifth is now being held here in Szeged under the title of the Fifth International Protoplast Symposium. It is particularly fitting that this Fifth Symposium should encompass all types of protoplast, because it provides us with the opportunity of drawing parallels and appreciating the differences between protoplasts from micro-organisms, algae, ferns, bryophytes and plants in general. Plant genetic manipulation will benefit greatly by such cross fertilisation of ideas; techniques and methodologies. It is also particularly fitting that this Symposium should be held in Szeged. Not only because of the steady ongoing work both in this Biological Institute on plant and bacterial protoplasts and in the University of Szeged on fungal protoplasts,but also because these studies epitomise the philosophy and aspirations of that great scientific son of Szeged, Albert Szent-Györgyi; and may provide us with an opportunity "To see what everyone has seen and think what no one has thought." (21).

The early work on plant protoplasts (see 2), was largely concerned with cytological studies. As early as 1939 Plowe (22), working with onion protoplasts stabilized in 0.56 M sucrose, was able to demonstrate the presence in such protoplasts of a morphological plasma membrane and tonoplast by microdissection and manipulation with glass needles. She was also able to demonstrate the differential permeability of both the tonoplast and the plasma membrane. This work extended to the Plant Kingdom the classical work of Chambers (1961) (23) on the nature of animal cytoplasm and membranes. It served to emphasise the basic similarities between cells at this level.

For many years plant protoplasts remained largely as cytological curiosities of little interest to physiologists, biochemists or geneticists. There was even a poem composed about protoplasts as physico-chemical systems (24). To my mind one of the most useful activities exhibited by protoplasts is that of cell wall regeneration. From my early studies on the short-term culture of enzymatically isolated tomato root protoplasts (25), it was concluded that it seemed likely that these protoplasts first develop some form of primary cellulose wall, and then, parallel to their later development they acquire some aspects of the behaviour of freely suspended isolated cells. This forecast was proved to be correct with the demonstration of wall regeneration by isolated tomato fruit protoplasts (26), and the subsequent demonstration that this could result in their sustained division (27).

The early studies on the division of *Haplopappus* protoplasts (28) showed that nuclear division (mitosis) was taking place while the protoplasts were remaining as naked cells, and before any wall regeneration had taken place. It was later observed that, if suitably cultured, *Petunia* leaf protoplasts would undergo nuclear division, giving rise to binucleate protoplasts, and that later, following wall regeneration they undergo both mitosis and cytokinesis (29,30). It seems likely that once wall regeneration has become initiated, mitosis and cytokinesis temporal relationships may be adjusted to each other, until the situation is similar to that normally found in cultured cells undergoing division (31,32). From these observations it was logical to expect that when a specific inhibitor of wall synthesis was available its effect on protoplasts would be to have no effect on nuclear division, but cytokinesis would be totally inhibited, so that multi-nucleate protoplasts would be obtained (33). These studies on cell wall regeneration by protoplasts have provided a wealth of new information, particularly on the early stages of wall synthesis and the

early organisation of the wall (34,35). These studies have also focussed attention on the nature of the plasma membrane in protoplast systems, and the importance of any glyco-calyx (36). Indeed, as far as fungal protoplasts are concerned, as recently discussed by Peberdy (37), this process of wall regeneration and subsequent reversion has been the aspect of fungal protoplasts most extensively studied so far. The use of bacterial protoplasts in genetic manipulation studies was held back by the apparent inability of protoplasts of Gram-positive bacteria to revert to the bacillary condition (12). In 1962 Landman and Halle re-discovered (38) in *B.subtilis*, what Nečas had already found in yeast in 1961 (39), that 25% gelatin medium was extremely effective in promoting reversion of protoplasts. As discussed by Landman and Castro-Costa (40) all bacterial strains that have been examined experienced difficulty in restarting a new wall once all the old wall has been lost. Clearly, when protoplasts give rise to L colonies consisting of wall-less irregularly shaped L bodies this condition has become a special heritable change. Whether such a condition could operate in fungi and plant protoplasts is, as yet, unresolved; but the recent studies of Herth and Meyer (33) on the cytology of budding and cleavage in dividing protoplasts are improving our knowledge in this area. Such colonies would be particularly useful in much the same way as the wall-less mutants of *Chlamydomonas* have proved to be (41).

Protoplasts, simply as protoplasts, offer an important biological entity for many physiological and biochemical studies as evidenced by, for instance studies on the photosynthesis of isolated protoplasts, protoplast extracts and chloroplasts of wheat (42), and by the ability to isolate guard cell protoplasts from onion and tobacco (43). The availability of protoplasts from guard cells and stomatal cell initials will allow more direct approaches to questions in stomatal development and function. Fractionation of protoplasts to provide the large scale isolation of mature plant vacuoles is now possible (44), and similar approaches have been used for the isolation of vacuoles from fungal protoplasts. As discussed by Cocking (2), the behaviour of protoplasts when subjected to a centrifugal force is of particular interest, and this behaviour is very reminiscent of the plasmolysis effects observed in certain elongated algal cells.Protoplasts of some species readily divide, without rupture of the plasma membrane into several smaller structures, called sub-protoplasts - the plasma membrane is apparently self sealing. Enucleated subprotoplasts (and nucleated subprotoplasts with a minimal amount of cytoplasm) have thereby been obtained with a minimum of disturbance to the organisation of the protoplasm, and at no stage has the plasma membrane been burst open (as it is when isolating vacuoles), exposing the cytoplasm directly to the external medium. Recently there has been renewed interest in the use of subprotoplasts mainly in relation to fusion studies (45), but they could also be used very meaningfully in assessments of the role of the nucleus in the control of cell wall synthesis in protoplasts. Protoplasmic units formed from protoplasts, but surrounded by an inner membrane of the protoplast probably distinct from the plasma membrane, are also of interest, particularly the relationship of the membrane surrounding these units to the membranes of the endoplasmic reticulum (46). Most of the current interest in these subprotoplasts and protoplasmic units has stemmed from the reconstruction of cells from cell fragments of animal cells (for a detailed discussion see (47)).

An attractive feature of bacterial and fungal protoplast systems is the readiness with which reversion takes place. This readily enables the

consequences of genetic manipulation at the protoplast level to be analysed. Certain algal and moss protoplast systems occupy an intermediary position in this respect. The situation regarding plant protoplasts is very variable ranging from failure to even divide, following wall regeneration, to the ready establishment, at high frequency of cell colony formation, and whole fertile plant regeneration. For plant protoplasts this is an area where much further work is required; and indeed it is lack of knowledge of the control of cell division in many species (particularly crop species) (48) which is delaying the application of the use of protoplasts for crop improvement. It is very encouraging that there is a suggestion that plant protoplast reversion can occur either via callus or embryoid formation depending on cultural conditions operating on a particular species (49,50).

The use of protoplasts for genetic manipulations centres on two major areas: one concerns the use of protoplasts in transformation studies, and the other the obtaining of genetic recombination or new genetic variability by protoplast fusion. Just as for Szent-Györgyi(21) his past successes (and failures) were never a hindrance to him - with confidence he pronounces, "I never look back, I only look forward," and just as for R.L. Stevenson, "To travel hopefully, is better than to arrive safely," so it is with investigators when using protoplasts for genetic manipulation!

The early work on the uptake of viruses and DNA by protoplasts has been well documented in several earlier reviews and key papers both for bacterial (12), fungal and plant protoplast systems(51,52,53), as has also work on protoplast fusion of bacterial, fungal, moss and higher plant systems (54). What will be most useful at this fifth landmark in the development of our understanding and utilisation of protoplasts is to try to assess what new dimension or perspective is arising from the use of protoplast methodology in this respect.

I think that Botanists can congratulate themselves on having provided, from this work on the fusion of plant protoplasts, a fresh impetus for the fusion of bacterial and fungal protoplasts and also even of animal cells. Hopwood (55) and Baltz (56) detected recombinant progeny in intra-strain mixtures of genetically marked *Streptomycete* protoplasts which had been allowed to regenerate after treatment with polyethylene glycol. More recently, ultra violet radiation of parental protoplasts immediately before fusion has been used to increase the proportion of recombinants (57). Szent-Györgyi would have been pleased to see such basic studies providing a novel approach in industrial strain improvement programmes! These studies were greatly facilitated by the ready availability of streptomyces derivatives carrying suitable genetic markers that could be used for complementation selection. This type of work on bacterial recombination by protoplast fusion was largely initiated by the pioneering studies in 1976 by Fodor and Alföldi (58), here in Szeged and by Schaeffer *et al* (59). These workers, with characteristic pre-science, saw from their studies using *B. megaterium* and *B. subtilis* that such bacterial protoplast fusions might be a new tool in bacterial genetics, providing diploid bacteria, or hybrids of bacteria. In more recent studies, on the polyethylene glycol induced fusion of bacterial protoplasts, Fodor and Alföldi (60), whilst able to directly select recombinants, noted a marked influence of the physiological condition of the protoplasts on recombinant frequencies.

As discussed by Peberdy (37) protoplast fusion in the filamentous fungi has been used to produce heterokaryons (which is a stable condition) between

strains of *Aspergillus nidulans* that are incompatible using conventional methods (61). In studies on *Cephalosporum acremonium* direct selection for presumptive haploid recombinants was employed (62) - protoplast fusion enabled parental nuclei to be brought into close proximity - an advantage because hyphal cells are mainly uninucleate and juxtapositioning of nuclei in such cells is infrequent. Protoplast fusion was facilitating the opportunity for parasexual recombination. Indeed after protoplast fusion these workers succeeded in breeding a strain of *C.acremonium* showing a 40% improvement in cephalosporin C titre. Somewhat similar recombination after protoplast fusion has been observed in the yeast *Candida tropicalis* (63). Parallel with these intra-specific studies has been an analysis of the use of fusion techniques in the production of inter-specific and inter-generic hybrids, and a major amount of this work has been carried out here in Szeged by Lajos Ferenczy and his co-workers (for a detailed discussion see (37)).As mentioned by Peberdy a meaningful explanation of the correct position regarding the nuclear interactions in these systems would seem impossible at the present time - it is possible that fusion products from any cross may yield subsequent progeny in which nuclear fusion and hence true hybridity has been achieved. In wider crosses, for instance, between *Candida tropicalis* and *Saccharomycopsis fibuligera* uninucleate strains showing varying degrees of instability have been detected. To a large extent these observations closely parallel the situation that has been found in relation to the fusion of plant protoplasts. Work on fungal protoplast fusion, just as has been the case for bacterial protoplast fusion, has been greatly aided by the ready availability of mutants that have enabled heterokaryons to be selected on the basis of nutrient complementation. This method of selection has also greatly facilitated the production of somatic hybrids by protoplast fusion in the moss *Physcomitrella patens* (64) and through somatic hybridisation the complementation analysis of auxotrophic mutants of this moss (65). When auxotrophic mutants are available they can be readily used for complementation selection of somatic hybrid plants (66). Protoplasts, being single cells, may be very suitable for mutant isolations and used for the improvement, for instance, of photosynthetic systems (67). From comparative studies in plant hybridisations of the incompatibilities that can be by-passed by *in vitro* culture methods in sexual hybridisation, with the hybridisation capability of somatic fusions, it is clear that pre-zygotic (68) and embryo/endosperm (69) incompatibilities can be readily overcome by somatic hybridisation. For plants, and also as we have seen for fungi (70) it is not yet clear whether the post-zygotic incompatibilities which prevent many wide inter species hybridisations also operate at the somatic level. Fungi differ markedly from plants in normally having a stable heterokaryotic condition which may enhance the opportunity for the specific control of nuclear fusion. As I have discussed recently (71,72) this comparative assessment of the nature of incompatibilities is made more difficult by lack of knowledge of the nature of inter-species sexual hybrid incompatibilities. Particularly whether or not it is pre- or post-zygotic, or both, and whether it can be circumvented by *in vitro* culture procedures; for instance by the rescue of embryos at a very early stage in their development, as has recently been employed by Williams in her production of *Trifolium* inter-species hybrids (73). Success in producing inter-species somatic hybrids should not lead to the conclusion that sexual hybridisation is impossible. Many interesting inter-species somatic hybrids are currently being examined in this respect. (74,75,76,77). A useful guide to incongruity of inter-specific crosses in *Nicotiana* and *Petunia* which could be of use to workers

generally in this comparative assessment has recently been outlined by Sink and Power (78).

It should be re-emphasised that there is a basic difference between sexual hybridisation and somatic hybridisation in that in sexual hybridisation there is usually unilateral exclusion of the cytoplasm - no such exclusion normally exists in the case of somatic hybridisation, since both somatic cell protoplasts contribute more or less equally to the cytoplasmic status of the resultant somatic hybrid. Protoplast fusion therefore makes possible the fusion of two different cytoplasms, allowing genetic analysis of cytoplasmic factors. In most instances so far investigated the fusion of higher plant protoplasts either results in the formation of somatic hybrid plants or in the formation of cybrid plants (see Fig. 1). Cybrid plants contain the cytoplasms of both parental species yet the nucleus of only one of the species. If nuclear fusion does not follow fusion of the protoplasts, and one of the nuclei degenerates, it is possible to obtain plants with hybrid cytoplasm and mixed organelles, but containing in its cells the nucleus of only one of the parents. Such cybrids could also be formed if the parasexual cycle is completed with complete directional chromosome elimination following the further mitotic division of somatic cell hybrids.

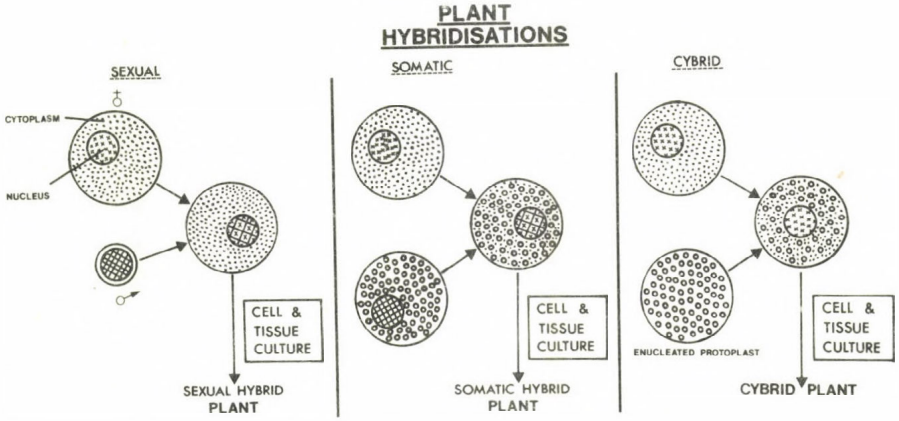

Fig 1. A schematic representation of the major procedures for plant hybridisation.

There is also considerable interest as to whether a complete parasexual cycle will be found to operate in the case of higher plants. As discussed previously, any tendency towards 'haploidisation' from somatic hybrids is most likely to occur in those species which exhibit a post-zygotic sexual incompatibility. Our earlier studies on the consequence of fusion of *Parthenocissus* protoplasts with *Petunia* protoplasts indicated that a significant feature of the callus obtained, as a result of the selective culture of fused protoplasts, was that callus was shown to possess the chromosomes of *Parthenocissus* and not *Petunia*. Discussing these results we noted that following fusion of chick erythrocytes with cultured mouse cells, a structural gene for chick inosinic acid pyrophosphorylase was incorporated into the mouse nucleus in some cells during post-mitotic

reconstitution. We suggested that a similar happening could be occurring as a result of the fusion of these post-zygotic sexually incompatible plant species; a result of which was that genes for *Petunia* isoperoxidases had become incorporated into the *Parthenocissus* nucleus(71). If somatic hybrid cells in culture undergo such changes, in many instances this could result in the production of cells (and plants) which possess a phenotype or genotype very similar to one or other of the parental species (79). Only very careful analysis of the genotype (both nuclear and cytoplasmic), as well as the phenotype, will reveal the extent to which such a parasexual-type cycle occurs in certain species combinations. In those species exhibiting pre-zygotic and embryo/endosperm incompatabilities it would appear that plant regeneration stabilises the somatic hybrids, but as wider crosses are attempted (and particularly those exhibiting post-zygotic incompatibilities) instability of the somatic hybrid may result in a tendency towards haploidisation (see Fig.2), with a novel genetic variability in the resultant parasexual-type hybrids.

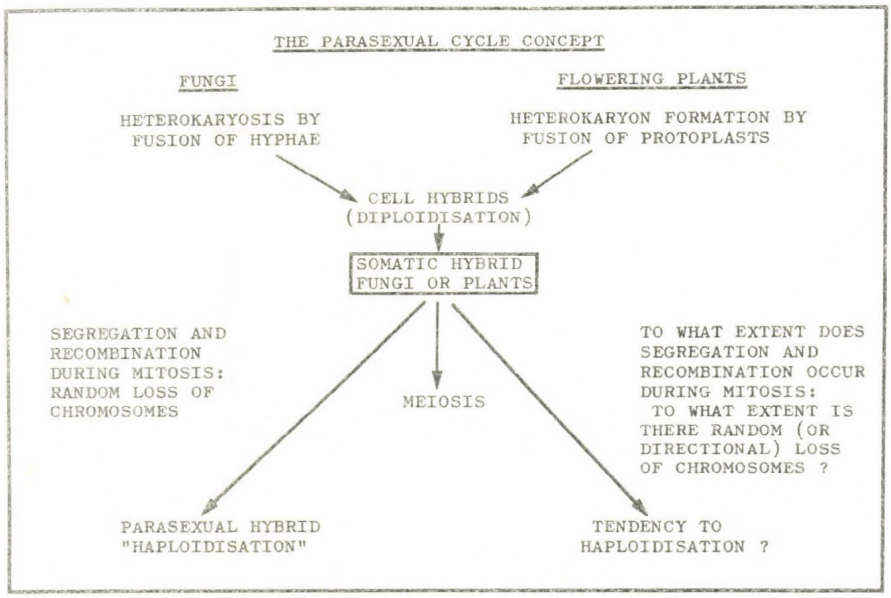

Fig. 2. The Parasexual Cycle Concept in Fungi and Flowering Plants.

Following the pioneering work of Ferenczy and his colleagues here in Szeged on the transfer of mitochondria by protoplast fusion in *Saccharomyces cerevisiae*, others (80) have further elaborated this study using mitochondria with drug resistance markers. The desire to use protoplasts for a more limited gene transfer could benefit from a comparative study of gametic transformation and somatic hybridisation. This recently discovered new technique of gametic transformation (81) offers the possibility of transferring single genes, or short genetic segments, from one species or variety to another in a single step without the simultaneous transfer of accompanying undesirable characters. In this method of sexual crossing, compatible pollen from a donor species is

treated with a high dose (100,000 rads) of ionising radiation and mixed with incompatible pollen before pollination. It will be of interest to determine, using protoplast fusion with one species irradiated, whether comparable transformation can be achieved. (see Fig.3).

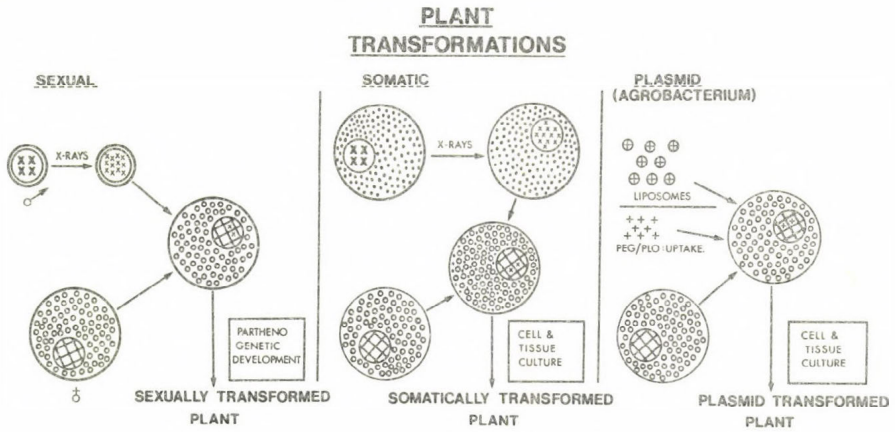

Fig. 3. A schematic representation of the three major procedures for plant transformation.

Plant protoplasts have provided us with a new *in vitro* system for plant virus research (82), but until recently there has been no unequivocal indication of the use of plant protoplasts for transformation work. The use of plant protoplasts to determine the fate of exogenous DNA has been critically evaluated (83), but the first clear indications that protoplasts of eukaryotic systems may be good systems for assessment, and proof, of transformation capability have come from work on yeast protoplasts. In these studies a hybrid bacterial plasmid containing yeast DNA was used as a source of highly enriched genes as well as a molecular probe from the sequence introduced into the recipient yeast cells. The behaviour of the bacterial plasmid DNA sequences, once integrated into the yeast chromosomes, indicated that prokaryotic DNA can be maintained and transmitted by a eukaryotic cell such as yeast (84,85).

As discussed by Kleinhofs and Behki (86) (see also (87)) the early work claiming exogenous DNA uptake, integration and replication in plants is probably wrong. Recently, however, the demonstration that isolated *Agrobacterium* plasmids can transform *Petunia* protoplasts clearly indicates that the future usefulness of plant protoplasts to be transformed by DNA vector consisting of T-DNA, and some advantageous genes, for the genetic engineering of plants, can now be seriously considered (87).

Work on the consequences of fusion of plant protoplasts with animal cells is still at an early stage of development, yet there is even already some evidence for cellulose being synthesised at the plasma membrane in such

heterokaryons (88), which can now be produced at high frequency following protease treatment coupled with Ca++ at high pH (89).

I have avoided speculating about the future and those areas of investigation are very much for future work. These inter-kingdom fusion experiments are however, important because they demonstrate the basic unity of Cell Biology. Work in this direction is difficult because it necessitates team work between plant and animal biologists.

If work on protoplasts can stimulate progress in all the various directions I have outlined it will have fully justified having a special meeting dévoted to this subject here in Szeged - and we can look forward to the Sixth International Symposium on Protoplasts with confidence, and some eagerness.

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MICROBIAL PROTOPLASTS

BACTERIAL PROTOPLASTS AND THEIR POSSIBLE USE IN BACTERIAL GENETICS

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ABSTRACT

Fusion of bacterial protoplasts is an established fact. The fused complex may be extremely sensitive to environmental influences. Mating type polarity does not exist in a protoplast fusion system of bacteria, the partners seem to be equal as far as their ability to donate or receive genetic information is concerned. True genetic recombinant bacteria can be obtained by fusion of protoplasts. Nevertheless, when the technique is used for genetic analysis the results are ambiguous. In order to be able to use the protoplast fusion technique for genetic analysis of bacteria more understanding of the interactions, both physiological and genetical, which take place during and after the fusion process is needed.

INTRODUCTION

Spherical forms appearing in cultures of rod-shaped bacteria were first noticed by Tomcsik and Guex-Holzer during their studies on the cell wall structure of *Bacillus* M. These entities seemed to be extremely labile and were rapidly disintegrated (1). Weibull working with another rod-shaped bacterium, *Bacillus megaterium*, had published in 1953 that if the cell wall of this bacterium is removed by a peptidoglycan hydrolysing enzyme, lyzozyme, in a medium with high osmomolarity, practically ever bacterium became spherical in shape. In the presence of high osmomolarity and Mg ions these spherical entities seemed to be quite stable and proved to be bacteria without a cell wall, surrounded only by a plasma membrane. The name *protoplast* was given by Weibull for these entities (2). McQuillen, in his study of protoplasts of *B. megaterium*, showed in 1955 that they can grow and divide in appropriate media (3). Finally Landman et al. in 1968 demonstrated that protoplasts of *B. subtilis* can be induced to revert into bacillary form (4).

The widespread use of protoplasts for genetic studies by fusion seems to start with the paper published by Kao and Michayluk in

1974 on the polyethylene glycol (PEG) induced fusion of plant protoplasts (5). PEG-induced fusion of fungal protoplasts was first published by Ferenczy et al. (6), PEG-induced fusion of bacterial protoplasts jointly by Fodor and Alfoldi on *B. megaterium* (7), and Schaeffer, Cami, Hotchkiss on *B. subtilis* in 1976 (8), PEG-induced fusion of *Streptomyces* protoplasts by Hopwood et al. in 1977 (9), PEG-induced fusion of yeast protoplasts by Sipiczky and Ferenczy (10) as well as van Solingen and van der Plaat in 1977 (11), PEG-induced fusion of *E. coli* protoplasts by Tsenin et al. in 1978 (12).

It should be mentioned here also that already in 1954 Stahelin published photographs of spontaneously fusing *B. anthracis* protoplasts (13). His observation remained completely unnoticed until recently.

It may be asked now why the fusion of bacterial protoplasts is interesting for bacterial geneticists? In order to be able to answer this question a few words have to be said about the genetics of bacteria.

Bacteria are haploid microorganisms, their genome being composed of closed circular double-stranded DNAs. There are several ways to transfer genetic information from one strain to other.

Transformation is called the process when genetic information is transferred from one bacterium to the other by means of naked DNA. The transfer of genes from one cell to another by means of bacteriophage vector is called *transduction*. *Conjugation*, or *mating* is a parasexual mode of unidirectional transfer of genetic information involving direct cellular contact between a donor ("male") and a recipient ("female") bacterial cell.

All of these processes exhibit at least three common features:
1/ The transfer is always unidirectional;
2/ DNA alone is transferred, so cytoplasm to cytoplasm interaction is not involved;
3/ a whole genome of a donor bacterium can seldom be introduced into a recipient one.

Finally, it has to be mentioned that the genetic information transfer mechanisms listed above operationally can be used only in a rather limited number of bacterial genera, therefore, a great many important bacteria are well without the possibility of study by a genetic approach.

It is immediately clear, therefore, that fusing bacterial protoplasts may add new dimensions to bacterial genetics. The way seems to be open to study the whole genome as well as cytoplasmic interactions in bacteria. Furthermore new genera of bacteria could be submitted to detailed genetic analysis.

For our studies we selected the Gram-positive, rod-shaped bacterium - *Bacillus megaterium* (7). This bacterium was also the object of early studies on bacterial protoplasts and we easily found the conditions were protoplasts can be reverted into bacillary form with high frequency (14, 15). Nevertheless, no genetic information transfer mechanism existed for that bacterium and our own preliminary attempts to attain transformation, transduction, or conjugation with it were without any success.

So the system seemed to be appropriate for showing what the fusion of bacterial protoplasts, if at all possible, can be used in bacterial genetics.

At the same time, independently from us, Pierre Schaeffer selected for his studies on the fusion of bacterial protoplasts the Gram-positive, rod-shaped bacterium, *Bacillus subtilis* (8). For him the advantage of this bacterium seemed to be its well studied genome, and the wealth of data already accumulated on it from transformation and transduction studies.

Our presentation will be concentrated on the data obtained with the *B. megaterium* protoplasts, nevertheless, we are going to refer to results obtained on *B. subtilis* by Schaeffer and his group.

EXPERIMENTS

The first figure shows how a fusion system should work. When working with bacteria the first thing to be demonstrated is that the recombinants are produced via protoplast fusion and not by any other possible information transfer mechanism. Under our experimental conditions recombinant bacteria of *B. megaterium* were obtained only when PEG-treated protoplast mixture was used. Furthermore, recombinants were obtained in the presence of DNA-ase, too. The conclusion was, therefore, that fusion of protoplasts is the mechanism which yielded the recombinants (7). The same conclusion was reached from their genetic data on *B. subtilis* protoplasts by Schaeffer, Cami and Hotchkiss (8). An elegant way to demonstrate protoplast fusion of bacteria with electron microscopy was recently published by Schaeffer and his co-workers (16). So fusion of bacterial protoplasts seems to be an established fact.

Let us analyse now what the characteristics of the system are:

a/ Cytoplasmic interactions. When bacterial protoplasts are fused by PEG treatment a new entity is produced, containing the genomes and cytoplasms of the parents.

It may be imagined that when parental protoplasts' membranes are disrupted on union the content of the protoplasts will be freely mixed. Such a free mixing, however, is probably not permissible; the fine structure and integrity of each cytoplasm has probably to be maintained.

Nevertheless, cytoplasmic components with low molecular weight, simple molecules, signal molecules, and so on, can be freely exchanged. The fate of the complex at a physiological level will be determined primarily by these interactions, which will then be further modulated by environmental-cytoplasmic interactions. Under these conditions the complex has to manage to revert into bacillary form. It is not surprising, that the fused complex may be extremely sensitive to environmental influences (17).

Conclusion to be drawn: Physiological influences may disturb the genetics of the system (17).

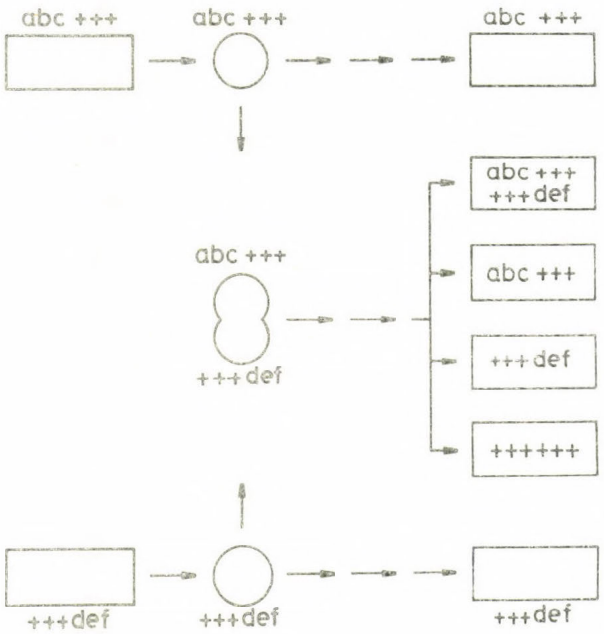

Figure 1 Schematic diagram of the steps of protoplast fusion

b/ Polarity. In the bacterial conjugation system there are donor ("male") and recipient ("female") bacteria, and an effective genetic information transfer involves unidirectional transfer of DNA from the donor into the recipient. Transfer is not possible in the other direction. Furthermore, recipient to recipient transfer is also impossible. The donor's characteristic is due to a so-called fertility factor.

Schaeffer's group and ours, respectively, wished to know if such a mating type polarity existed in the fusion system of protoplasts.
Schaeffer's group found that before fusion any one of the *B. subtilis* parental protoplasts could be inactivated by streptomycin and that recombinants could be obtained if the other parent happened to be living and streptomycin resistant (18). Our group found that before fusion any one of the *B. megaterium* parental protoplasts could be inactivated by gentle heat treatment, and that recombinant bacteria could be obtained if the other parent's protoplasts were alive (19).

The conclusion is clear : mating type polarity does not exist in a fusion system of these bacteria, the partners seem to be equal as far as their ability to donate or receive genetic information is concerned.

Nevertheless, the answer is not so clear when we ask if, in the formation of the recombinants, both genomes are playing an equal role.

c/ Detection of fusion recombinants. In order to detect fusion recombinants two main methods can be followed:

1/ Plating the PEG-treated mixture of protoplasts on reversion medium rich in nutrients. This will allow reversion to bacillary form parentals and recombinants. Selection of recombinants can be made then from that medium by means of the classical replica plating onto different selection media (indirect selection).

2/ The PEG-treated mixture of protoplasts is plated directly onto reversion media supplemented according to the aim of the selection. Such a medium allows only the outgrowth of recombinant colonies (direct selection).

True genetic recombinant bacteria can be obtained by both techniques. Nevertheless, it has to be emphasized here that, as we mentioned earlier, the outgrowth of recombinant bacteria from the fused protoplasts is strongly influenced by the physiology of the system. In other words, one never knows if a genetic event which is possible in principle on a given medium will yield the expected type and expected number of recombinants, or if they will not be present at all because of physiological factors inhibiting or preventing reversion to bacillary form by that fusion product.

d/ Genetic analysis. In order to understand the genome structure of a bacterium the first step is usually to construct its genetic map. The genetic map is a representation of relative distances separating non-allelic gene loci in a linkage structure, the distances being measured on the basis of recombination frequencies. Linkage analysis of three genetic markers is the most common way to arrange the order and give the map distances for the given markers, which when extended step by step to new markers may yield a complete linear or closed circular linkage map.

Standard genetic maps are made under standard conditions optimal for crossing over and hence for recombination. Unfortunately, in a fusion system with bacterial protoplasts the first condition to be taken into consideration is how reversion to bacillary form can be ensured. Nobody knows at present how these peculiar conditions influence the recombination system. Corinne Levi in Pierre Schaeffer's laboratory has recently shown, working with two *rec*$^-$ strains of *B. subtilis*, that in fusion experiments the same results were obtained as with the *rec*$^+$ strains (20). So, either the recombination system is not needed to yield recombinants via protoplast fusion, or in the *rec*$^+$ strains the recombination system is inhibited by the experimental conditions.

In our recent studies with protoplasts of *B. megaterium* we have considered that recombinants selected for a given marker and permitted to be recombined for two other markers ought to

give useful linkage data when the experimental conditions are strictly standardized. Furthermore, we have simplified the system to a one-way genetic information transfer by inactivating the wild type +++ parent by streptomycin, as was described by Levi et al. for B. *subtilis* (18). The other parent, triple auxotroph and streptomycin resistant, was used in that type of cross as a *recipient*; therefore, in selecting for any one of the transferred wild type alleles a single nutrient had to be omitted from the reversion medium. Furthermore, a selection medium always permitted the free expression of the other two markers of the donor. So, on any selection medium four genotypes were able to be expressed under exactly the same conditions: +--, ++-, +-+, and +++, the phenotypes of each being identifiable by a second step progeny analysis.

This type of analysis has as its advantage that predictions can be made as far as the frequency of the non-selected markers is concerned as seen on the Figure 2. In principle, the order of the studied markers and their linkage ought to be deduced from the experimental data.

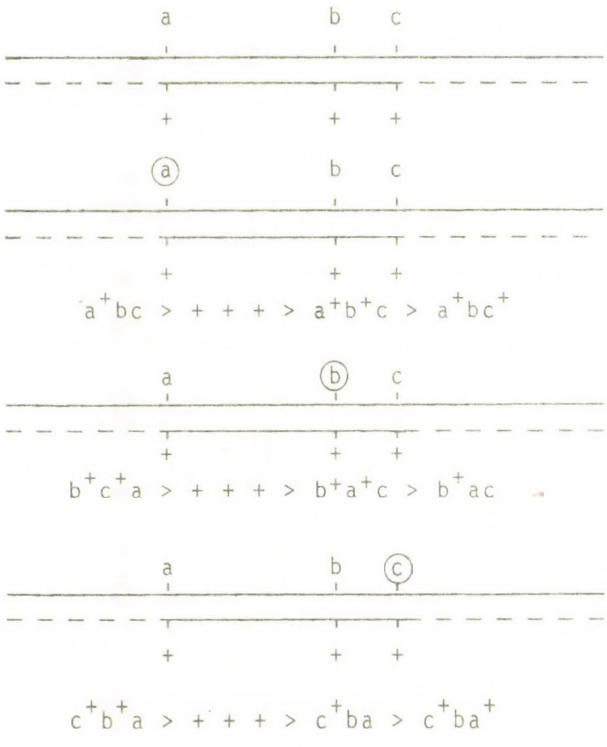

Figure 2 Expected distribution of markers at different selection

Table 1 shows how the experiments worked. Between repeated experiments considerable differences may exist. Nevertheless, as far as reasoning about linkage relationships is concerned some preliminary conclusions can be drawn. The supposed linkage data can then be tested using new combinations of the markers. These experiments are under way in our Laboratory.

There are anomalies also present in our results. The relation of the +++ and +-- categories seems to be, for example, quite unpredictable.

TABLE 1

B. megaterium THT Str^R (Try$^-$, His$^-$, Thr$^-$) x *B. megaterium* KM Str^S (Streptomycin-killed)

	EXPERIMENT		
Try$^+$ selection	1	2	3
Try$^+$ His$^+$ Thr$^+$	55	80	42
Try$^+$ His$^-$ Thr$^+$	6	2	1
Try$^+$ His$^+$ Thr$^-$	9	0	1
Try$^+$ His$^-$ Thr$^-$	30	18	56
Total:	100	100	100
His$^+$ selection			
Try$^+$ His$^+$ Thr$^+$	48	12	28
Try$^-$ His$^+$ Thr$^+$	26	13	12
Try$^+$ His$^+$ Thr$^-$	3	1	1
Try$^-$ His$^+$ Thr$^-$	21	13	58
Total:	100	100	100
Thr$^+$ selection			
Try$^+$ His$^+$ Thr$^+$	63	83	61
Try$^-$ His$^+$ Thr$^+$	24	12	12
Try$^+$ His$^-$ Thr$^+$	1	1	0
Try$^-$ His$^-$ Thr$^+$	9	3	25
	3	1	2 mixed
Total:	100	100	100

The numbers represent colonies with the given phenotype.

Order of markers suggested: His Thr Try

DISCUSSION

The system we have presented here for genetic analysis at first sight seems to be simple. Since the ability of one of the parents to revert into bacillary form and yield bacterial colonies is inactivated by streptomycin, the genetic information transfer ought to be unidirectional. Furthermore, appearance of the non-selected markers ought to depend only on genetic factors since physiological factors on a given selection medium are the same for every fusion product.

Nevertheless, contrary to these expectations, the results are ambiguous, it would be hard to arrive at any clear-cut conclusions from these results. So the question can be raised: Is the system really as simple as it seems at first sight?

The first question to be discussed is if the inactivation one of the parents may influence the outcome of a cross in a non-predictable way. Streptomycin is known to interfere with ribosomal functions, but DNA of the inactivated parent should not be altered. Ribosomal functions of the other parent are streptomycin resistant; therefore they should function in a normal way even if bound or free streptomycin happens to remain in the system after washing the streptomycin out before fusion. Nevertheless, cytoplasmic interactions might play some role by influencing the fate of the fusion product. We do not know, for example, if the cytoplasm of the inactivated parent might not be "reactivated" by the cytoplasm of the other parent. These bacteria would be then detected as +++ wild type "recombinants". When tested, the great majority of these wild type recombinants proved to be streptomycin resistant which suggested that they are real recombinant progeny of the recipient parent. Nevertheless, streptomycin sensitives are also often detected among these wild types, and with them it is impossible to decide if they are reactivated parentals or recombinants which received the streptomycin sensitivity as a non-selected marker, too.

The second question to be raised may be if, even under identical conditions, some classes of the recombinants are not handicapped by physiological factors and therefore the distribution of non-selected markers is not the expression of linkage relations in genetic terms but the expression of linkage relations in physiological terms. A convincing answer cannot be given to this question at present.

The third disturbing factor may be in the technique of detection of the phenotypes of the selected colonies. Namely, the population of a selected colony may be composed of individual segregants with different phenotypes (7). In order to test the phenotype of a colony a suspension is made from it (or replica plating is carried out), where even from of a majority population of a minus phenotype, individual segregants with + phenotype will grow on every medium. Therefore, without further steps in progeny analysis one never can be sure that a + phenotype of a colony is true or whether one is dealing with a mixed population. Till recently we were convinced that at least

the minus phenotypic markers can be accepted and used for linkage analysis without any doubt, but after having read the paper of Rollin Hotchkiss (21) one will realize that we might be wrong even in this respect.

Taking all the above discussed facts into consideration, one has to realize that further efforts are needed to eliminate the uncertainties in the system. Furthermore, it is quite clear that to use the protoplast fusion technique for genetic analysis one needs more understanding of the interactions, both physiological and genetical, which take place during and after the fusion process.

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RECOMBINATION, SEGREGATION, AND UNEQUAL CHROMOSOME EXPRESSION IN BACTERIAL DIPLOIDS CREATED BY PROTOPLAST FUSION

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In the normally haploid bacteria, although fragments of one genome had often been introduced into another cell by various means of transfer, total genomes were first brought together by protoplast fusion. When fusion of *Bacillus subtilis* protoplasts was first announced by Schaeffer, Cami and Hotchkiss (1), they reported the formation of haploid genetic recombinants showing new combinations of markers derived from two different auxotrophic parent lines. This recombination was frequently multiple within the tiny fraction (10^{-4}) of the cells which showed recombination at all. Since all prototrophs and recombinants appeared to be haploids, it was inferred that any diploid stage (which would be expected to be prototrophic through complementation) that resulted from fusion must be short-lived. Under the conditions used at that time usually less than 10 % of input protoplasts were being successfully regenerated into viable bacterial colonies, therefore to detect recombinants it was necessary to sample massed bacterial regenerants obtained from around 10^6 protoplasts. It was thus quite possible to imagine that the entities giving rise to recombinants might be some relatively unusual intermediate, e.g. a polykaryotic aggregate created by the exposure to the polyethylene glycol (PEG).

Fodor and Alföldi, in an accompanying paper (2), had reported similar yields of recombinants from doubly auxotrophic *Bacillus megaterium* parents, with some evidence of unstable intermediates giving rise to the recombinants and, sometimes, parental types. They allowed genetic selection to operate already during the regeneration process (2,3), which was adequately complete under their conditions.

Subsequent reports have described evidence that one parental protoplast culture - either one - can be killed, with streptomycin (4) or by heat (5),without seriously disturbing the genetic consequences of what must be essentially an interaction of whole chromosomes. Hopwood and associates have meanwhile reported that frequent multiple recombinations occur in *Streptomyces coelicolor* fused in protoplast form (6).

At this point it was easy to suppose that for the *Bacillus* species successful fusion was an occasional event, but recombination

was a very frequent occurrence in those occasionally fused cells. We began to doubt this only when several signs appeared that fusion was actually frequent in itself.

We had succeeded in improving the experimental conditions so that virtually all *B. subtilis* protoplasts can be regenerated, and so that even after PEG treatment, 50-75 % of them could form bacterial colonies (7). Using these conditions we found that fusion was frequent enough that recombination was not limited by collisions between protoplasts (not a second or higher order power of protoplast concentration) but was a simple linear function of the total protoplast concentration. If one parent was "diluted" into a larger quantity of another protoplast type, the proportion of minority type participating in recombination actually increases (7) - the excess of the other protoplasts ensuring fusions in proportion to the total number of protoplasts present. Nevertheless, only the usual low yield of recombinants was obtained. It appeared that some factor other than collision and contact limited the occurrence of genetic recombination.

Through the friendly cooperation which has been maintained between the Orsay laboratory and our own, we learned at an early time that Sanchez-Rivas and coworkers had demonstrated considerably more directly that fusion events were frequent in *B. subtilis* protoplasts treated with PEG. The work has just been published, together with our own, just summarized. Electron microscope study (8) with prespores as cell markers showed that 2- and 3-member fusion products bounded by a single membrane were formed during a few minutes incubation following PEG treatment. The number of cells involved in fusion could be estimated as reaching 10 to 50 % of the protoplasts present. In a parallel work (9), phage complementation between two induced lysogenic parents was used to measure fusion without requiring the process to continue through to cell wall regeneration. In this way again it could be estimated that 20 to 50 % of the population took part in fusion, one-half of which involved heterologous pairs and could yield infectious phage.

Thus, from one-tenth to one-half of the protoplasts became fused, by all accounts, yet prototrophs had been detected only as part of the tiny fraction making haploid recombinants. We had to suppose that the great majority of the fused heterologous pairs must have segregated into parental cells before they were able to produce colonies of prototrophic diploids. But, we reasoned, since we are now able to regenerate virtually all of the protoplasts, we should find a sign of the temporary existence of the heterodiploids - what we predicted as "biparental" colonies. These would be colonies which after regeneration could be demonstrated to contain both of the two differently marked parental auxotrophic types, reflecting their transitory existence in some complex resulting from fusion.

Looking, then, not at the massed regenerating cells on crowded plates, but at the separated colonies derived from high dilutions of the post-fusion mixture, we easily found that some 1 to 5 % of randomly picked recovered colonies contained such a mixture. A few, one percent of the total, more or less, proved to be genetic recombinants, constituting 10-50 times more than

could be detected among the massed regenerants. The rest of the colonies could be classified as either one or the other of the parents althouth separate studies showed that even a few of these are recombinants which have acquired one or two wild-type genes from an opposite parent. Typical early experiments and the principles upon which they are based are given in Table 1.

TABLE 1 Primary regenerated colonies from fusion of multiple auxotrophs

Cross : Parent A $(ALM)^-$ x Parent B $(T\theta U)^-$

Primary regenerated colonies	Growing in medium A	Growing in medium B	Growing in both A and B	Growing in neither A nor B
Definitions:				
Parent A	+	0	0	no
Parent B	0	+	0	no
Biparental AB	+	+	+	no
Certain recombinants	0	0	0	yes
Experimental findings:				
I 439	238	216	18	3
II 671	383	297	15	7
III 336	189	162	15	0

Symbols: A, adenine; L, leucine; M, methionine; T, threonine; θ, tryptophan; U, uracil ($^-$ mutants showing deficiency) As strains and medium, A signifies parent A which grows in ALM or medium A, a minimal medium supplemented with adenine, leucine and methionine.

The biparental mixed cultures may be supposed to arise from early segregation of the fused heterologous pairs, which from the work cited above are expected to have formed in considerable numbers. On the other hand, they might be merely the reflection of protoplast pairs simply sticking together, or aggregated, without having fused, at the time of sampling. But, it turned out that many of the biparentals continue to behave as biparentals and still give rise to mixed cultures after repeated passages. This is indicated schematically in Table 2, in which one biparental (I), is shown as separating before the first test merely behaving as a mixture, another (II) has separated by the time of its third passage, while III still appears biparental after the fourth passage. As indicated, either parent, A or B, can "carry" the other parent as A(B) or B(A), as it continues to grow on its selective medium, and these two types are found in approximately equal proportions, and have similar ranges of stability. The biparental state can continue for as many as 8 or

TABLE 2 Behavior of biparental colonies on further propagation

Colony type and number	Sampled from medium	Ability to grow on selective media				Diagnosis
		minimal	A	B	complete	
Biparental I	regen.	0	+	+	+	AB
" AB-I	A	0	+	0	+	A seg.
" AB-I	B	0	0	+	+	B seg.
Biparental II	regen.	0	+	+	+	AB
" AB-II	A	0	+	0	+	A seg.
" AB-II	B	0	+	+	+	B(A)
" B(A)	A	0	+	0	+	A seg.
" B(A)	B	0	+	+	+	B(A)-2
" B(A)-2	A	0	+	0	+	A seg.
" B(A)-2	B	0	+	+	+	B(A)-3
" B(A)-3	A	0	+	0	+	A seg.
" B(A)-3	B	0	0	+	+	B seg.
Biparental III	regen.	0	+	+	+	AB
" AB-III	A	0	+	+	+	A(B)
" AB-III	B	0	0	+	+	B seg.
" A(B)	A	0	+	+	+	A(B)-2
" A(B)	B	0	0	+	+	B seg.
" A(B)-2	A	0	+	+	+	A(B)-3
" A(B)-2	B	0	0	+	+	B seg.
" A(B)-3	A	0	+	+	+	A(B)-4
" A(B)-4	A	0	+	+	+	A(B)-5

Comments: Illustrative examples are represented of three different biparental colonies (I to III) taken directly from regeneration medium. Diagnosis of successive later passages (indicated by arabic numbers), from the medium indicated in the second column, leads to the conclusion that I has already segregated when first tested; that II has segregated by the third passage; and that III is relatively stable being still biparental at least at a fourth passage. The identification as A(B) or B(A) cannot be made until a test is made in the first passage. Each type of behavior shown has been observed in over a hundred colonies, with minor variations.

more passages, through at least 100 cell generations. The experiment presented in Table 3, representative of many others, indicates that approximately equal numbers, around one percent of the regenerated colonies, or slightly more, appeared in the following three classes: (a) early recombinants, (b) biparentals

which segregated more or less early , and (c) biparentals which persisted for many cell divisions, some of them producing late recombinants. The recombinants thus recognized among single colonies, and even the small group of late recombinants were far in excess of those detected in the usual way by replica plating from massed regenerants.

TABLE 3 Single colony analysis of fusion V: $(ALM)^-$ x $(T\theta U)^-$

Among 1032 post fusion colonies there were found:

27 biparental colonies (2.6 %)
14 early recombinants (1.4 %), including ALθ, ALMU, LMTU and 5 others

From the 27 biparental colonies:

15	had segregated pure parents at	25 divisions
10	still carried two parents at	50 "
2	gave one parent and one recombinant at	50 "
6	still carried two parents at	70 "
1	gave two parents, one recombinant at	70 "

(Late recombinants: 3 colonies, 0.3 %)

Standard replica plating gave only 0.057 % recombinants.

What is most important and unexpected about the biparentals is that they manifest uniquely the phenotype of only one of the parents, although it can be either one. It is quite uniformly true that they require for growth precisely the three nutritional factors required by that parent (and not merely one, or two, of the three), and that they remain independent of those three needed by the other parent. Yet, after segregation that other parent will reappear, showing in full its requirements and now manifesting its independence of the very factors which the biparental had required. The more stable biparentals, at least, must be considered as diploids in some sense, since they can propagate as such, segregate, and show recombination. It is most challenging, therefore, that they do not show the expected complementation between the genomes they carry.

It will be important to determine in what way the two genomes coexist during the persisting biparental state. It might be thought that one parental culture, growing on its own medium, can, by cross-feeding the other, supply required nutrients sufficiently well to maintain it. We have proceeded along two lines of investigation which show this to be unlikely: reconstruction experiments, and subcloning. First, no mode of cocultivation of two strains, among the several tried, succeded in preserving a parent on the medium selective for the other; cross-streaking, mixing and serial transfer in different ways on different media, all revealed that instead of cross-feeding, the strains were rather severely competitive against each other, when growing at close range. Even with mass passages of several

hundred cells on selective solid media, a mixture becomes pure for the selected parent in one passage or at most two. Any remaining doubt disappeared when we subcloned the biparental cultures after a substantial number of generations in rich liquid medium, which would separate or dilute out any artifactual aggregates if they should be present. Even though the process involved three passages (growing, spreading a dilution, and regrowing clones), an appreciable proportion of the cultures still contained biparental clones. Since we have not yet fully explored the effect of various media on the stability of biparentals, we may not have obtained optimal conditions for preserving them.

An illustration of what can be observed among 1000 randomly chosen regenerants after fusion is given in Table 4. About 15-20 % of the original biparentals are generally found to be of the "stable" type, persisting at least in part as diploids through the several passages on complete or rich medium involved in subcloning. A larger proportion persist through such passages on solid media as those described in Table 2, where a larger inoculum is passed to media selective for the phenotype being expressed. A second subcloning tends to show a high proportion of diploids in the preselected clones. Except for the approximate one percent of genetic recombinants gradually appearing in the diploid subclones, the phenotypes detected in biparentals correspond precisely to those of one, or the other, of the original parents. Several crosses with different parental strains have given this same result.

TABLE 4 Processing of post-fusion colonies : summary of experience

Random regenerated colonies		Screen 1000 colonies	
Growth in one parental medium	gives	Parents, A or B	900-950 col.
" " both "	media	Biparentals	50
Solid selective media; (30 div.)		Segregated parents	38
		Persisting biparentals	12
Liquid rich media; cloned (35 div.)		Parents, A and B	45
		Diploids	5
Screen diploids in:			
Parental medium A		A(B) + few A	2-3
Parental medium B		B(A) + " B	2-3
Minimal medium 0		Diploid prototrophs:	0.005
Selective media various		Recombinants var.	0.05

UNEQUAL CHROMOSOME EXPRESSION IN BACTERIAL DIPLOIDS

Survival of a culture bearing one phenotype through numerous passages and cloning, the reproduction and retention of an unexpressed genotype in some cells, and the moderately frequent appearance of recombinants between the two genomes, lead us to conclude that PEG-induced diploids are being succesfully propagated in these normally haploid bacteria. Evidence for another kind of diploid has in the meantime been discovered, among a fraction of the relatively rare prototrophs found at 10^{-5} level during recombinant screening. By searching among fused rec-cultures for prototrophs giving rise to auxotrophs, Levi (10) was able to find some of them - four clones were examined in detail - which segregated different stable auxotrophs while being propagated as a prototroph. Furthermore, Sanchez-Rivas (11) has observed that rec+ strains also give rise to such segregating prototrophs at similar frequency if appropriately selected. The segregating types indicated in the original *B. megaterium* work may be quite similar, also representing a rare kind of diploid in which the two genomes do complement each other (2).

The diploids which we have found and described above seem to be produced at a level about 100 times more frequent. It therefore appears that two chromosomes can be, but only exceptionally are, expressed within the same bacterium. This is quite unexpected, in view of the extensive work with partial diploidy in bacteria, brought about by transfers of plasmids, episomes, chromosome fragments and other DNA entities, and their ready, if not inevitable, expression. Although whole chromosomes transferred during fusion, like such fragments, can often be subsequently replicated, the very completeness of protoplast fusion may be what permits us to observe the possibility that they can assume and be maintained in an unexpressed state. The persisting habit of expression of the one selected chromosome in any given diploid line, as described, suggests a physical organization or association (with membrane,etc.) which is different for the two chromosomes, and epigenetically propagated. This possibility will be a subject of our continuing investigations. It might be interesting to inquire whether in other fusion systems, a large class of similar non-complementing diploids may be overlooked in the screening of fusion products.

SUMMARY

Conditions devised for virtually complete regeneration of bacterial colonies from poly-auxotrophic *Bacillus subtilis* protoplasts have permitted a quantitative analysis of single colonies resulting from protoplast fusion. As previously observed, only a small yield (less than 10^{-4}) of prototrophic bacteria are obtained, most of them haploid recombinants. A much larger number, 2 to 10 percent, of the regenerated colonies are biperantel, containing the genomes of both parents in unchanged form. Many of these have already separated, or soon segregate into typical parental cell lines, but a substantial proportion can be cloned as diploids stable through as many as 90 cell generations. Unexpectedly, their phenotype during the diploid phase is precisely that of one or the other parental type, and not the assumed

prototrophic one which would result from complementation. It appears that the bacterial chromosome can assume a configuration or association in which it can be replicated, but is not transcribed or expressed. About one percent of intergenome recombinants of a variety of types appear among the first post-fusion colonies, and a similar proportion of recombinants are found in diploid clones at a later time when they have segregated into haploids.

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TRANSFER OF PLASMIDS INTO BACTERIAL PROTOPLASTS

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ABSTRACT

The transfer of non-transmissible plasmids pSF2124, pBR322, pMB9 and pJJ1 between different *E. coli* F⁻ strains has been demonstrated by the method of polyethylene glycol-induced fusion of protoplasts. The plasmids that have been transferred by this method were maintained steadily in the great majority of the recipient bacteria. A study of the transfer of plasmids between cells of systematically distant microorganisms is under way.

A new, recently developed technique for the transfer of genetic information between the cells of microorganisms is polyethylene glycol-induced fusion of protoplasts. This technique was used to obtain recombinant forms of different strains of *B. subtilis* (1), *B. megaterium* (2), *Streptomyces* (3). The possibility of protoplast fusion and the formation of recombinants for *E.coli* has been shown by Zenin et al. (4). The aim of this work was to investigate whether protoplast fusion could be used for transferring of non-transmissible plasmids between different *E. coli* strains.

E. coli cells were grown on a minimal medium with 10 % sucrose and other necessary factors at 30°C. Protoplasts were obtained according to the method of Weiss (5) modified by Zenin et al. (4) (lysozyme, 100 µg/ml; EDTA, 0.01 M, pH 7.0 in the presence of deoxyribonuclease 5 µg/ml and $MgCl_2$, 0.05 M). The number of viable cells in the suspension of protoplasts varied from 0.5 to 10 % depending on the strain.

Equal volumes of two suspensions were mixed, incubated for 20 mins at room temperature, centrifuged and resuspended in a 40 % solution of PEG to obtain the initial volume. The samples were put into 0.6 % agar and plated either onto an agar medium enriched with Casamino acids (0.5 %) and yeast extract (0.5 %) or onto a minimal medium with the amino acids necessary for the

recipient strain. The protoplasts of recipient strains only were also plated as a control. After 5-6 days of cultivation at 30°C the soft agar layer was scraped off, resuspended in an appropriate liquid medium and cultivated for 18-20 hrs. Then the samples were plated onto an agar nutrient broth with antibiotics. The cultivated colonies were examined for determinants of recipient cells and plasmids.

The characteristics of the strains and plasmids are shown in Table 1.

TABLE 1 Strains and plasmids of *E. coli*

E. coli strains	Genotype	Plasmids
NF59	F⁻met arg relA str-r	
GC146	F⁻pro his metA malB lac gal str-r	
C600 (pSF2124)	F⁻leu thr B1	pSF2124 Ap, El^{1mm}, synthesis El
C600 (pBR322)	F⁻leu thr B1	pBR322 Ap, Tc
C600 (pJJ1)*	F⁻leu thr B1	pJJ1 Ap, Km
C600 (pMB9)	F⁻leu thr B1	pMB9 Tc

*The plasmid pJJ1 was constructed in the Laboratory of A.I. Stepanov in the Institute of Genetics and Selection of Industrial Microorganisms in Moscow. It consists of whole molecules of pBR322 and pUB110 and determines the resistance to kanamycin and ampicillin in *E. coli* cells.

Using the method described above, we successfully transferred all the plasmids shown in Table 1 into the cells of the recipient strains. When $1\text{-}3\times10^9$ cells were plated onto the selective media, the average frequency of appearance of colonies containing the plasmids was about 10^{-7}, though in some experiments it reached high values (see Table 2). This frequency was the same when the mixture of protoplasts was plated both onto the enriched agar medium and onto the minimal agar medium containing the

factors which were necessary for the cultivation of the recipient cells only.

TABLE 2 The frequency of appearance of the colonies on selective media

Donor	Recipient	Antibiotics	Frequency of appearance of the colonies*
C600 (pSF2124)**	NF59	Ap, Str	1×10^{-7}
-	NF59	Ap, Str	$< 1 \times 10^{-8}$
C600 (pBR322)	NF59	Ap, Tc, Str	5×10^{-8} - 1×10^{-7}
-	NF59	Ap, Tc, Str	$< 1 \times 10^{-9}$
C600 (pBR322)	GC146	Ap, Tc, Str	6×10^{-8} - 1×10^{-7}
-	GC146	Ap, Tc, Str	$< 3 \times 10^{-9}$
C600 (pJJ1)	NF59	Ap, Km, Str	1×10^{-7} - 1×10^{-4}
-	NF59	Ap, Km, Str	$< 2 \times 10^{-9}$

*The frequency is estimated relative to the number of cells per ml after cultivation in the liquid medium. The concentrations of the antibiotics were: ampicillin - 20 μg/ml; tetracyclin - 20 μg/ml; streptomycin - 90 μg/ml; kanamycin - 20 μg/ml.

**In this experiment the control was the transfer of plasmid between the intact cells of the donor and the recipient: no plasmid transfer was found.

The frequency of appearance of the colonies containing plasmids was low. We selected cells containing plasmids pBR322 and pJJ1, respectively, on plates with two antibiotics, so they were not antibiotic-resistant mutants. In the case of plasmid pSF2124 the ability of recipient cells to synthesize colicin E1 was also determined.

An additional period of cultivation in a liquid medium was required because, when the cells were plated onto selective media immediately after having been cultivated in soft agar, no plasmid-carrying colonies were found.

It should be noted that the frequency of transfer varies substantially depending on the condition of the protoplasts of both strains and on the number of viable cells in the protoplast suspension.

The plasmids that have been transferred by this method could be maintained steadily in the most isolated clones for 40-80 generations.

We also tried to carry out the direct selection of plasmid-containing recipient cells in the experiment with protoplast fusion of *E. coli* C600 (pBR322) and *E. coli* (NF59).

After the mixture of the protoplasts had been kept in 40 % PEG, the suspension was diluted 5fold with the cultivation medium containing DNAase and $MgCl_2$ (0.05 M) and incubated with aeration for 2 hours at 30°C. The samples were then plated onto the enriched agar medium with tetracycline (20 µg/ml) and streptomycin (90 µg/ml). In 4-5 days, the colonies of the recipient were observed on this selective medium, and the frequency of their appearance, relative to the number of the recipient protoplasts reverted into the cell form, was equal to $1x10^{-6}$.

To determine whether the transformation of *E. coli* protoplasts by the plasmid DNA is possible under these conditions, the protoplasts of *E.coli* NF59 (obtained without DNAase) were incubated with 5 µg DNA pBR322 in the presence of 40 % PEG for 20 min. Then PEG was removed by centrifugation, the protoplasts were suspended in a fresh, enriched cultivation medium and incubated with aeration for 1.5 hrs at 30°C. No colonies with plasmid-resistance determinants were observed in the experiments with direct selection (i.e., when the protoplasts were plated onto the agar containing tetracycline and streptomycin) or in the experiments without direct selection which were carried out as described above. These experiments show that under these conditions the transformation of *E. coli* protoplasts by plasmid DNA does not occur.

Thus, the possibility of plasmid transfer between cells of different strains of *E. coli* by the PEG-induced fusion of protoplasts is shown. This technique can be used for the transfer of non-transmissible and non-mobilizable plasmids.

At present, we have begun experiments to study plasmid transfer between the protoplasts of systematically distant microorganisms. We have tried to transfer a number of plasmids from the cells of *E. coli* (RP4, F104, F150, F126) into *B. megaterium* by using the protoplast fusion technique, but no plasmid genes have been expressed so far in the cells of *B. megaterium*.

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USE OF SPHEROPLASTS TO TRANSFORM YEAST WITH CHIMERIC PLASMIDS

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INTRODUCTION

Yeast transformation has been shown possible by Hinnen et al. (1) with a yeast LEU2 gene carried by a bacterial vector. The frequency of transformation was very low (10^{-7} transformants per regenerated cells). As yeast is an eucaryote and genetically well characterized, it is potentially a good system for the transformation and cloning of foreign DNA sequences. It is, therefore, important to construct a range of possible vectors and selection systems. Beggs (2) used the 2 µm yeast plasmid, in which the LEU2 gene was inserted, the whole being linked to pMB9. Such a plasmid transformed yeast with a frequency of 10^{-4}.

We developed such a host-vector system with chimeric plasmids containing the URA3 gene and the 2 µm yeast DNA linked to the bacterial plasmid pCR1. Yeast transformation is achieved with a high frequency and the URA3 gene is maintained on a cytoplasmic element (3).

MATERIAL AND METHODS

Strains. The *S. cerevisiae* recipient strain was ura 3.160-288, which bears two mutations in the URA3 gene and was constructed by Losson+. The *E. coli* pyrF is devoid of orotidine 5' monophosphate decarboxylase (OMP decase) and this mutation can be complemented by the yeast URA3 gene (4).

DNA preparations. were performed according to classical methods (5,6).

Construction of chimeric plasmids. The 1.1 Kb URA3 gene (which codes for the OMP decase) was extracted from a clone obtained from a gene bank (7) and characterized by Bach et al. (4). PTY 39 DNA (i.e. 2 µ cloned in pCR1) was given to us by Hollenberg (8). We inserted the URA3 sequence in PTY 39 at Hind III sites.

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Transformation of yeast. Cell collected, in log phase were converted into spheroplasts with snail enzyme (6500 units/ml, at a cell density of $2x10^8$ cells/ml in 1.2 M sorbitol, 0.05 M citrate phosphate buffer pH 5.8) in about 1 hour. After centrifugation and washing with 1.2 M sorbitol, spheroplasts were concentrated at 10^9 cells/ml in 1.2 M sorbitol, 10 mM Tris, 10 mM $CaCl_2$ pH 7.5. Plasmid DNA was mixed with 0.2 ml of the spheroplast suspension to a final concentration of 5 to 15 µg/ml. 2 ml of polyethylene glycol 4000 (30 % in 10 mM Tris, 10 mM $CaCl_2$, pH 7.5) were added and after 15 min. the spheroplasts were centrifuged and allowed to incubate in complete medium (1.2 M sorbitol, 4 g/l yeast extract, 6 g/l glucose, 6 g/l peptone, 10 mM $CaCl_2$, 10 mM Tris pH 7.6) for 1 hour at 28°C. After centrifugation, cells were resuspended in 0.2 ml of the preceding medium and mixed with 10 ml of regeneration agar (1.2 M sorbitol, 20 g/l glucose, 0.8 g/l tryptone, 0.3 g/l yeast extract, 6.7 g/l yeast nitrogen base, 30 g/l Difco purified agar) at 44°C and poured on plates of the same medium (with only 20 g/l agar).

Regeneration was estimated by the ratio of the number of colonies growing on the same medium supplemented with uracil to the number of spheroplasts counted in the cell suspensions.

Agarose gel electrophoresis, hybridization of DNA were carried out according to classical methods (9, 10).

Orotidine 5' monophosphate decarboxylase assay. The crude extract of cells grown on minimal medium were obtained essentially following Lacroute (11) and enzyme assay was conduced as in Beckewith et al. (12).

RESULTS

1. Plasmids used for transformation. We have used three types of plasmids, the structure of which is given in Figure 1. G10-53 is identical to G9, except the bacterial part which is pBR 322 instead of pCR1.

2. Efficiency of transformation. The regeneration of spheroplasts is very critical for the transformation process. Therefore, we did not try to get true protoplasts but rather spheroplasts (i.e. osmotic sensitive cells). We also avoided the use of thiol compounds. The DNA treated spheroplasts were submitted to an "expression phase" in an osmotic rich medium; we observed that this step had a positive effect on regeneration. Regeneration itself took place in solid medium with 3 % purified agar (which solidifies only at 42°C). Some yeast extract was also added at a concentration (300 mg/l) sufficient for yeast cells to undergo the first divisions needed for the expression of the ura^+ phenotype. On the control plates no colonies appeared under our experimental conditions.

With a regeneration rate of 3 to 25 %, the transformation frequency (per viable cell) varied between $2x10^{-5}$ and $2x10^{-4}$.

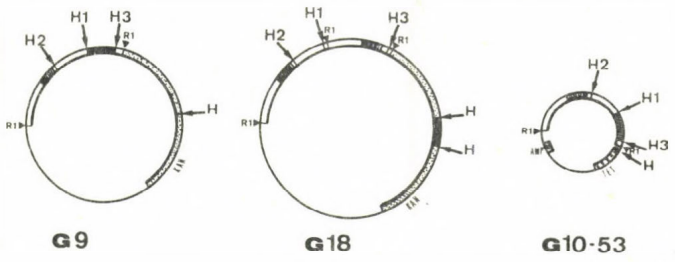

Figure 1 - Structure of chimeric plasmids

Kanamycin resistance gene; URA3 gene;

Inverted repeated sequences; 2 µm;

Tetracycline resistance gene; Ampicillin resistance gene;

Eco RI site; Hind III site

In G9 and G18 the bacterial part (——) is pCR1 ; in G10-53, it is pBR 322.

3. Presence of the G9 plasmid DNA in yeast transformants.

(a) Hybridization tests were carried out with total DNA of transformants (hydrolyzed with Eco RI) using ^{32}P labelled G9 DNA as a probe (Figure 2, part I) of ^{32}P pBR 322 ura 3 (Figure 2, part II)

The 2.2, 2.4, 3.8, and 4.0 Kb bands correspond to the 2 µm DNA (for review, see ref. 13), the 13.5 and 5.1 Kb bands to the G9 vector; the 12.0 Kb band to the Eco RI fragment of the chromosome which bears the URA3 gene, and the 3.5 Kb band can be explained by recombination events. As can be seen, the transformants had the bands corresponding to G9 and the 12.0 Kb band is unchanged in transformants compared to the recipient strain. Thus, it does not seem that an integration of the URA3 gene took place.

(b) Recovery of plasmids. Circular DNAs were extracted from yeast transformants and used to transform *E.coli* pyr F. The bacterial transformants had the ura^+, kan^R phenotype. This result shows that the URA3 gene is at least in part carried by an autonomous replicon in yeast.

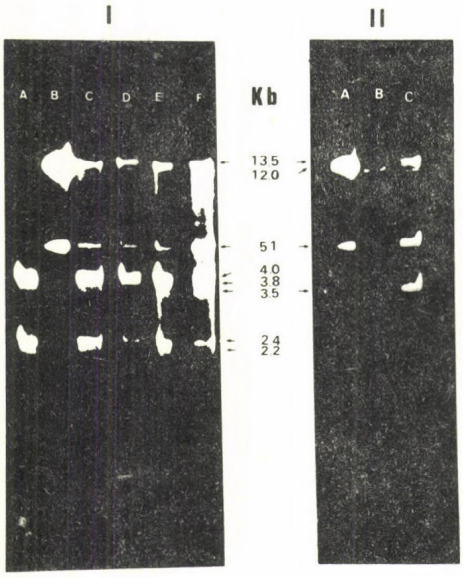

Fig. 2. Autoradiogram showing hybridization of ^{32}P-labelled DNA with Eco RI digest of various strains.

(in part I, A corresponds to the recipient strain, B to G9 DNA, C,D, E,F, to transformants; in part II, A corresponds to G9 DNA, B to the recipient strain, C to a transformant)

4. Genetic analysis. Transformants are unstable and give rise after culture on minimal medium to about 40 % of ura$^-$ clones. On supplemented medium this instability is somewhat enhanced (80 % ura$^-$). Successive transfers on minimal medium did not result in an increase of stability. If ethidium bromide is added to the minimal medium (at a final concentration of 0.5, 5 or 50 µg/ml) the growth of the transformants is identical to that of the wild type on the same medium. This suggest that ethidium bromide does not interfere with the expression of the URA3 gene or with the replication of the plasmid.

The diploids obtained by crossing the transformants with a ura3 strain are auxotrophs or prototrophs and in this latter case are also unstable. Finally, the segregation pattern of the ura$^+$character is in meiosis non-mendelian; there is an

excess of the 0:4 and 4:0 tetrad types, and the average transmission of the ura$^+$ character is between 20 % and 42 %. These results show that URA 3 is maintained as a cytoplasmically inherited element.

5. OMP decarboxylase. The specific activity of this enzyme in yeast cells transformed with G9 (or G18) is 30fold (or 15fold, respectively) the activity measured in the wild type. The URA3 gene carried on the hybrid plasmids is thus very well expressed in yeast.

DISCUSSION

The vectors presented here are maintained and replicated in yeast and in *E. coli*. Two of them (G9 and G10-53) are composed of two parts, bacterial and "yeast". The latter is the 2 µ-URA 3 sequence, which can easily be removed from the bacterial part and ligated to any chimeric plasmid in which Eco RI sites are available. Thus, this is a valuable tool to study the expression of cloned genes in yeast. Struhl et al. (14) have now published results also showing that the 2 µm plasmid efficiently increases the transformation frequency in yeast. However, they observed an unstable integration of the yeast gene introduced, which is not the case in the URA3 system.

SUMMARY

Chimeric plasmids have been constructed, bearing the bacterial plasmids pCR1 or pBR 322, part or all of the 2 µm yeast plasmid and the URA3 yeast gene. These plasmids transform *S. cerevisiae* with high frequency. No evidence of integration into the chromosome has been found. Hybridization tests, genetic studies and the recovery of plasmid from yeast transformants suggest a cytoplasmic inheritance of the vector. The cloned URA3 gene is very well expressed in yeast. Recombination events take place between the 2 µm DNA and the vector.

ACKNOWLEDGEMENTS

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INCORPORATION OF ALGAL THYLAKOID MEMBRANE AND DNA IN YEAST PROTOPLASTS

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SUMMARY

Protoplasts released from Saccharomyces cerevisiae were strongly aggregated with photosynthetic organelles isolated from a blue-green alga, Anabaena cylindrica, or from a green alga, Chlorella ellipsoidea, by polyethylene glycol-calcium chloride. These organelles were incorporated into the vacuole of the protoplast as thylakoid fragments or disintegrated materials through a "protostoma" formed at the bottom of the protoplast invagination. No intact algal photosynthetic organelle was found in the yeast protoplast. Further, as a result of algal DNA uptake, a possibility of increasing reversion in yeast protoplast (Ade) was supposed.

INTRODUCTION

In a previous report (1), we suggested that intraspecific fusion of yeast-protoplasts is accomplished through a break in the junction of plasma membranes. The incorporation of an intact algal chloroplast into the protoplast of a higher plant has been found by H.T.Bonnett and M.S. Banks (2). In yeast, transfer of mitochondria by protoplast fusion has been genetically proved by L.Ferenczy and A.Maráz (3), and also transformation in protoplasts by using polyethylene glycol has been reported by A.Hinnen, J.B.Hicks and G.R.Fink (4). The process of uptake of algal photosynthetic organelles, blue-green algal thylakoids or green algal chloroplasts, and of algal DNA into yeast protoplasts,and reversion of the yeast protoplast occurring during the process are reported in this paper.

MATERIALS AND METHODS

Preparation of yeast protoplasts: Protoplasts were released from exponentially growing cells of Saccharomyces cerevisiae (HUT 7135 and a mutant, Ade$^-$, induced from IFO 1136) by 1 % Zymolyase-5000/0.6 M KCl/50 mM Tris-HCl, pH 7.2 (5).

Preparation of thylakoids: Cells of Anabaena cylindrica (IAM M-1) during growth in modified Detmer's medium were ruptured by osmotic shock (2 to 0.6 M sorbitol). Thylakoids were isolated by centrifugation, and then stored in 0.9 M sorbitol/50 mM Tris-maleate, pH 6.0.

Preparation of chloroplasts: Exponentially growing cells of Chlorella ellipsoidea(IAM C-102) in modified Bristol's medium were suspended in 1M sorbitol/2% Ficoll-400/60mM phosphate, pH 7.2, and agitated with glass beads(0.5mm diameter). Chloroplasts were fractionated by centrifugation at 205000xg for 1 hr in a sorbitol gradient(1 to 4 M) dissolved in 2% Ficoll-400/60 mM phosphate, pH 7.2 (2).

Aggregation of yeast protoplasts and isolated organelles: A mixture of the protoplasts and the thylakoids(1:1) was incubated in 0.9M sorbitol/10mM $CaCl_2$/20%(w/v) polyethylene glycol MW 7500(PEG)/50mM tris-maleate, pH 6.0, at 20°C for 20 min (1, 6, 7, 8). For chloroplast-protoplast aggregation(30:1), 1M KCl /20% PEG/2% Ficoll-400/60mM phosphate, pH 6.0, was used. For morphological examination, incubation was 1 to 4 hr.

Isolation of algal DNA: DNA was isolated from each of the cultures of A. cylindrica and C. ellipsoidea by Marmur's method after the disruption of cells (as before) in 0.15M NaCl/0.1M EDTA (9).

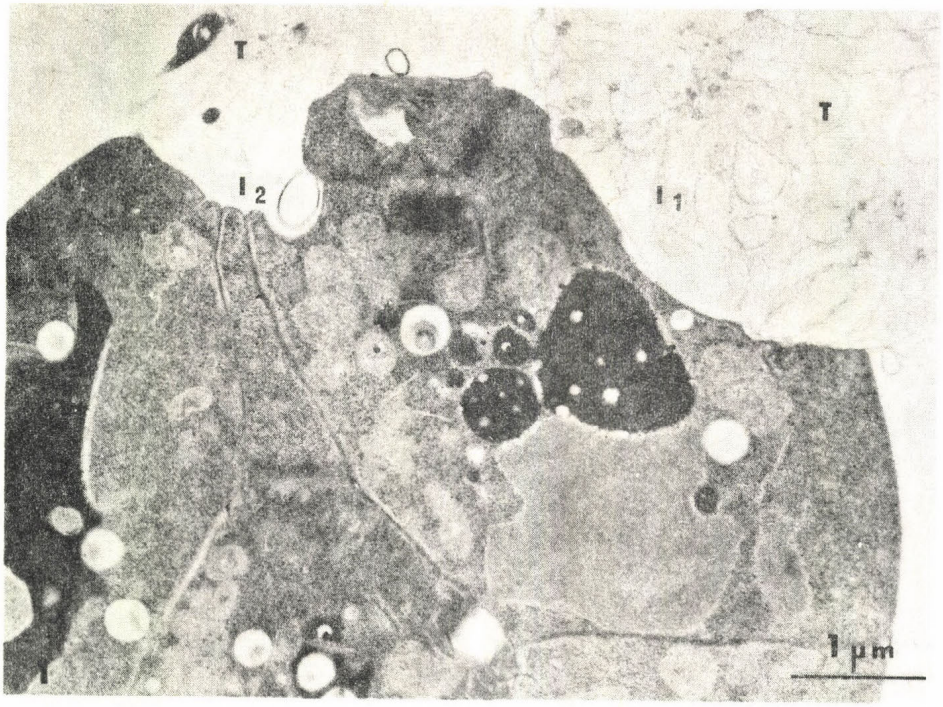
Uptake of DNA: Mixtures of 1 ml 20% PEG/0.6M KCl/10mM $CaCl_2$/ 50mM tris-maleate, pH 6.0 (1), and 0.1 ml of 150mM NaCl/15mM Na-citrate containing each DNA corresponding to about 0.5 g(wet weight) of cells were added to each of the sediments of yeast protoplasts(about $2x10^8$), and subsequently incubated at 20°C for 20 min after agitation.

Regeneration of protoplasts: Aggregates were spread on regeneration agar plates containing 2% malt extract/0.2% yeast extract/2% sucrose, or Burkholder's medium, and then covered with 10 ml of 0.6M KCl/20% gelatin (10).

Specimen preparation for electron microscope examination: Aggregates were fixed with about 3% glutaraldehyde by adding 25%(in water) reagent to each aggregation media. After the glutaraldehyde fixation at 20°C for about 6 hr, the aggregates were post-fixed with O_sO_4, dehydrated, embedded, sectioned and stained according to the method as described in the previous report (1).

Fig. 1. Aggregate of Saccharomyces cerevisiae protoplasts and Anabaena cylindrica thylakoids. A protoplast has an invagination(I_1) curved along the surface of the thylakoid(T). The other invagination(I_2) is formed by more than 4 protoplasts.

Fig. 2. Saccharomyces cerevisiae protoplast incorporating a thylakoid of Anabaena cylindrica. The thylakoid(T) membrane with an increased density by disintegration near the invagination(I) is incorporated through a protostoma(PS) and stored in a vacuole(V) as a highly dense material.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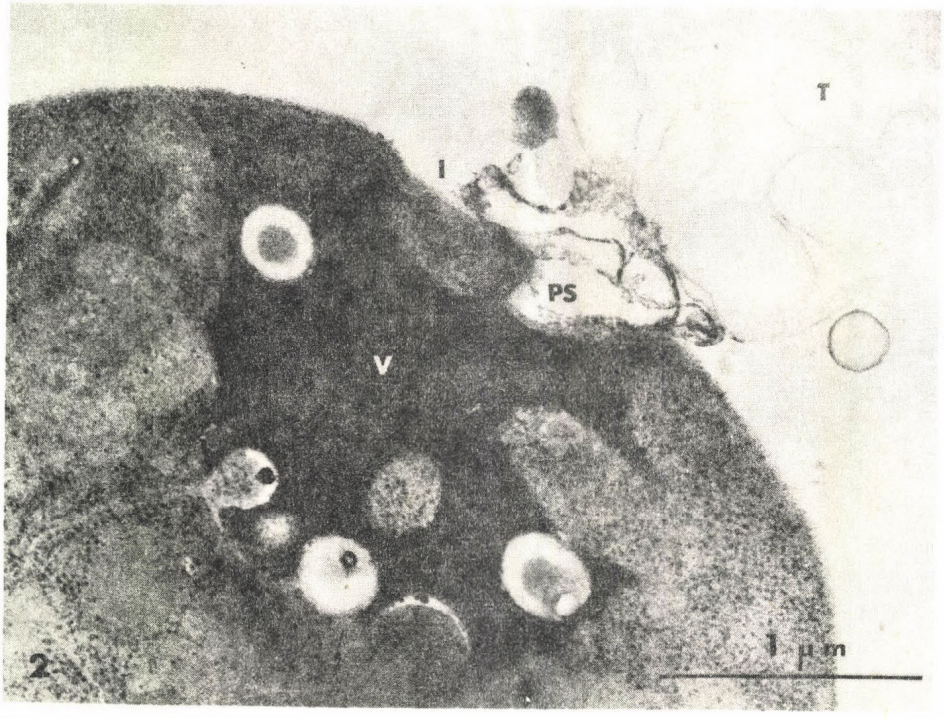
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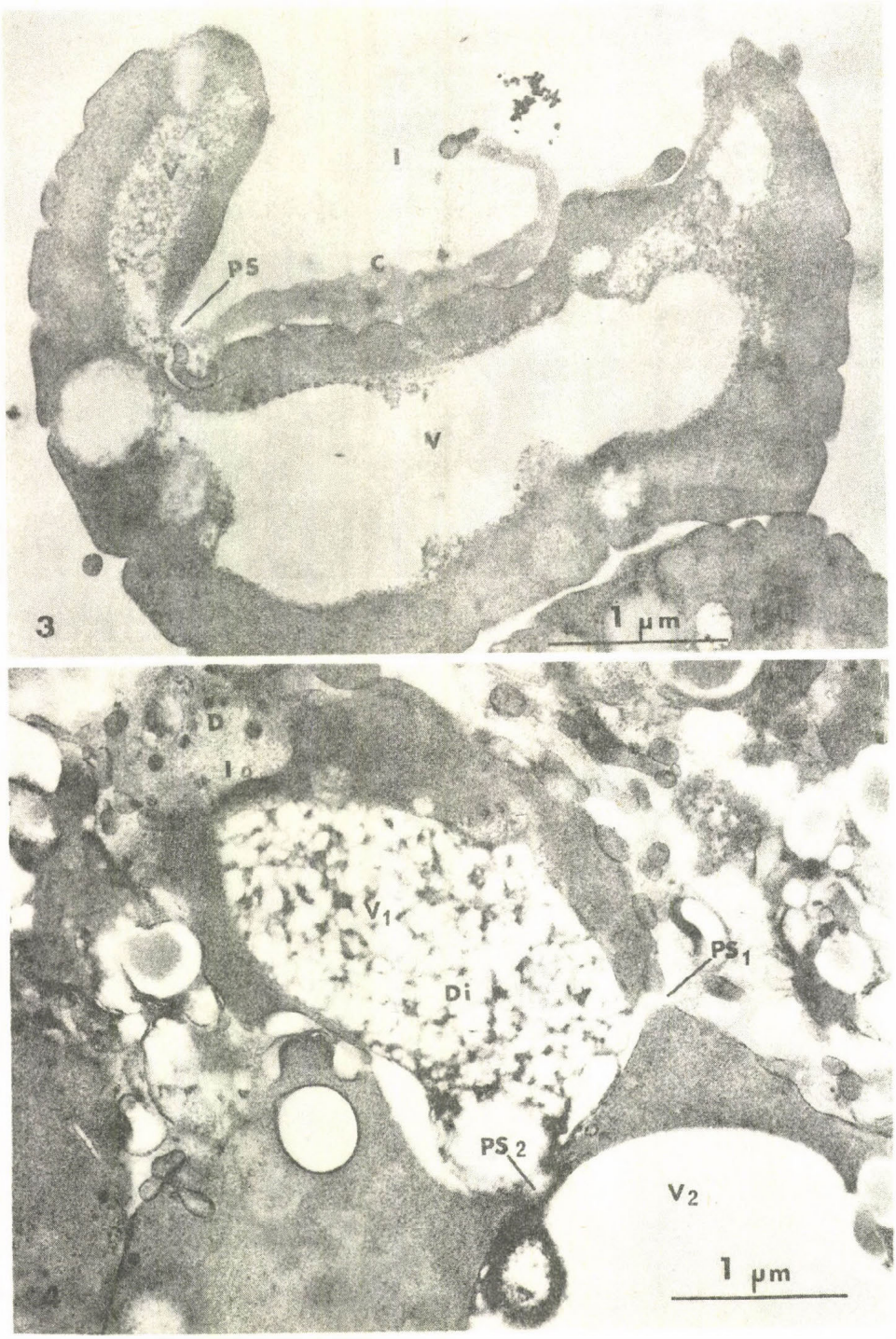
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Table 1. Reversion of Saccharomyces cerevisiae Ade⁻.

Intact cells	Proto-plasts	DNA AC	DNA CE	T	C	PEG	$CaCl_2$	Regene-ration	Reversion fr. ($x10^{-3}$)
+	-	-	-	-	-	-	-	-	<0.0016
-	+	-	-	-	-	-	-	+	0.27
-	+	-	-	-	-	-	+	+	0.22
-	+	+	-	-	-	-	-	+	2.5
-	+	+	-	-	-	-	+	+	3.4
-	+	-	-	-	-	+	+	+	0.42
-	+	-	-	+	-	+	+	+	3.4
-	+	+	-	-	-	+	+	+	10.3
-	+	-	+	-	-	-	-	+	<0.12
-	+	-	+	-	-	-	+	+	0.49
-	+	-	-	-	+	+	+	+	0.25
-	+	-	+	-	-	+	+	+	1.8

Abbreviation: AC=Anabaena cylindrica, CE=Chlorella ellipsoidea, T=thylakoids, and C=chloroplasts.

RESULTS AND DISCUSSION

Uptake of algal photosynthetic organelles in yeast protoplasts: Yeast protoplasts were so strongly aggregated with thylakoids or chloroplasts that they could be seen as green aggregates of protoplasts by light microscopy. However, no intact thylakoid or chloroplast was found in the yeast protoplast by electron microscopy. The yeast protoplast formed an invagination at the adhesion surface between the protoplast and these organelles(Figs. 1, 2, 3). The protoplast could form several invaginations, and also an invagination was made by several protoplasts(Fig. 1). A "protostoma"(a mouth of protoplast) occurred at the bottom of the invagination(Figs. 2, 3). Membrane fragments transferred from these organelles near the protostoma (may have been digested by enzymes localizing on the protoplast surface) were incorporated into the protoplast, and then stored in the vacuole(Figs. 2, 3). On the other hand, the yeast protoplast was able to engulf the intact cells of C. ellipsoidea protected by a rigid cell wall.

Fig. 3. Saccharomyces cerevisiae protoplast incorporating a chloroplast of Chlorella ellipsoidea. The chloroplast(C) squeezed by PEG and disintegrated is incorporated through a protostoma(PS) and stored in a vacuole(V) as a mass of fragments.

Fig. 4. Aggregate of Saccharomyces cerevisiae protoplasts with DNA extract from Anabaena cylindrica. The extract(D) is inserted between protoplasts and caught in the invagination(I). The extract(D) is, then, incorporated into a vacuole(V_1) through a protostoma(PS_1). The incorporated extract(Di) is transferred again into the other vacuole(V_2) in the adjacent protoplast through the other protostoma(PS_2).

Uptake of DNA into yeast protoplast: The same process of incorporating A. cylindrica DNA through the protostoma as described above was demonstrated in Fig. 4. Reversion frequencies of S. cerevisiae (Ade^-) obtained under several conditions are shown in Table 1. The reversion was inducible through only protoplast formation, regardless of aggregation of PEG and calcium chloride. There was no reversion (less than about 10^{-6}) in the intact cell growth. Contaminant DNA in the incubation medium for protoplast formation presumably caused some reversion, because, as as possibility, Zymolyase-5000 used for the protoplast formation might contain a trace of DNA from S. carlsbergensis, which was used as a substrate for the enzyme production by Arthrobacter luteus (by a private communication from Dr. K. Kitamura, Kirin Brewery Co. Ltd.). However, the highest frequency (about 1 %) of reversion was attained when the mixture of the yeast protoplasts and DNA of A. cylindrica yielded by spooling was aggregated with PEG and calcium chloride. In the uptake of C. ellipsoidea DNA, the reason for low frequency is still unknown.

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FUSION OF PROTOPLASTS OF AUXOTROPHIC FUNGAL MUTANTS: DIVERSITY IN THE GENETIC BACKGROUND OF NUTRITIONAL COMPLEMENTATION

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In experiments aiming at fusion of fungal protoplasts auxotrophic parental mutants are employed in most cases and the resulting nutritional complementation is indicative of protoplast fusion. Recognition or selection of fusion products is based upon the prototrophic or partially complemented nature of the resulting colonies and, in addition, the fusion frequency may be calculated by comparing the number of colonies developing in nutritionally incomplete medium to that growing in a complete one. Of course, the frequency of protoplast fusion and the frequency of nutritional complementation can never be the same since fusion also occurs between identical (non-complementary) partners.

Whatever methods are used for protoplast formation and fusion (1), one of the final tasks is to characterize the fusion products and to determine the nature of nutritional complementation.

In the past few years a considerable mass of data has accumulated on variations in the genetic background of nutritional complementation as a consequence of protoplast fusion. The aim of the present contribution is to review this diversity.

INTRASPECIFIC PROTOPLAST FUSION

Heterokaryon Formation

The first successful experiments concerning controlled fungal protoplast fusion were carried out with auxotrophic mutants of *Geotrichum candidum* (2, 3). In these mutants sexual or parasexual processes have never been observed. Protoplast fusion was induced by centrifugal co-sedimentation of protoplasts of auxotrophic mutants followed by long incubation. Complementation occured with low but constant frequencies. Serial transfer of the complemented hyphae could be performed indefinitely on minimal medium. Complementation, however, was temporary. The complemented hyphae gave rise to auxotrophic arthrospores.

Similarly, heterokaryon formation is the only known genetic process resulting in complementation in *Phycomyces blakesleeanus* auxotrophic strains (4). The fusion frequency was also low, with the best results obtained in the presence of $Ca(NO_3)_2$ at pH 7.

In *Mucor racemosus* the prototrophic isolates that arose with comparatively high frequency after protoplast fusion of auxotrophs gave rise to sporangiospores of either parental phenotype and to prototrophs (5). Permanently complemented cells were not observed; after repeated transfer, prototrophic colonies continued to segregate spores with the parental phenotypes.

Heterokaryon with Occasional Stable Diploid Formation

Aspergillus (3, 6-11) and *Penicillium* species (7, 8, 10, 12) are the typical representatives of this class. In *Aspergillus nidulans* an increased frequency of protoplast fusion of auxotrophic mutants was obtained when natural aggregation occurred (6), and high frequency complementation resulted by using polyethylene glycol (PEG) as fusogenic agent (7-12) in the presence of Ca ions. On minimal medium mycelial growth could be maintained indefinitely. In general, these colonies which were irregular in appearance, gave rise to auxotrophic conidia of the parental types. Occasionally, vigorously growing, well-organized sectors developed. These sectors contained larger, uninucleate, complemented conidia with double the DNA content of either the parent protoplasts or the heterokaryons. Haploids of the parental and recombinant types could be induced from these diploid conidia or from the diploid mycelia.

Heterokaryon with Frequent Stable Diploid Formation

A characteristic species displaying this phenomenon is *Candida tropicalis* (13-15). From auxotrophic mutants heterokaryons, heterozygous protrotrophs, somatic segregants, and recombinants could be obtained. Interestingly, uninucleate prototrophs originating from diauxotrophs spontaneously gave rise to monoauxotrophs (13). This might indicate the existence of aneuploids. This idea is also supported by the low DNA content of certain prototrophs (14). If red adenine-requiring and white cysteine--requiring mutants were used in the fusion experiments, the multinucleate heterokaryotic prototrophs were coloured a wide variety of shades of pink and these readily gave rise to auxotrophic cells of the parental types even on minimal medium. The diploid (and/or aneuploid) prototrophs were white, rapidly growing, and genetically extremely stable (14, 15).

Heterokaryon with Frequent Transient Diploid Formation

In *Cephalosporium acremonium* heterokaryons were easy to obtain with a high frequency (10). However, heterozygotes were rarely

recovered. All heterozygotes proved very unstable on complete medium. Diploidization was transient and was often followed by rapid chromosome segregation and, possibly, intrachromosomal recombination (16),

Unstable Diploid Formation

Protoplasts of *Saccharomycopsis* (*Candida*) *lipolytica*, heterogenic for complementing nutritional markers but identical for mating type have been fused (17). The fusion gave rise to slow-growing diploid colonies and these were analyzed for recombination. Spore formation was induced in about 3 % of the diploid fusion products; all examined progeny were of (+) mating type as were the parental strains. These results confirm that mating type alleles of this species, in contrast to many other yeasts, control only the initial steps in the mating sequence.

Stable Diploid Formation

Using haploid auxotrophs of identical mating type, stable diploids could be constructed in a series of yeast species. Heterokaryon formation was so transient that these were not detected. The diploids were stable; usually spores were not produced. The fusion cells were uninucleate, enlarged, and their DNA content was about twice that in the parental haploid cells. By induced haploidization or by crosses with cells of the opposite mating types both haploids with the parental markers and recombinants could be recovered. If protoplasts of opposite mating types were fused, the fusion products showed the characteristics of normal crosses. The following organisms display the above phenomena: *Schizosaccharomyces pombe* (18-20), *Saccharomyces cerevisiae* (19-27), *Rhodosporidium toruloides* (28), *Kluyveromyces lactis* (29-31), *Hansenula wingei* (20).

In addition to diploids, cells of lower and higher ploidy and different types of aneuploids were also occasionally observed as a result of the fusion of haploid protoplasts (32-36). Even multiple fusion leading to triploids and tetraploids may be encountered. Triploid fusion products have been formed by protoplast fusion of two different strains as well as three different strains (27).

INTERSPECIFIC PROTOPLAST FUSION

Heterokaryon and Diploid Formation

Aspergillus nidulans and A. *rugulosus* are closely related species. Their fusion products showed several of the characteristics of products obtained by intraspecific protoplast fusion in A. *nidulans*. However, differences were also observed. The slowly growing heterokaryotic colonies gave rise to vigorously developing diploid sectors which, however, suffered from slightly

disturbed regulatory processes. This was indicated by the reduced amount of conidia with abnormal structure of the conidial apparatus, the presence of anucleate small conidia among the bigger diploid ones and by the secretion of a brown pigment (37). Conidia of the diploid colonies were heterogenous for the auxotrophic markers. Analyses of induced segregants revealed a high degree of chromosomal homology between the two partners (38).

The fusion of protoplasts of the closely related *Penicillium chrysogenum* and *P. cyaneo-fulvum* auxotrophs yielded rather similar results (39), and essentially the same conclusions can be drawn as those above. On the basis of these and more recent similar findings it seems worthwhile to re-evaluate the taxonomic boundaries and interrelationships of several fungal species and groups.

Heterokaryon and Heteroploid Formation

If protoplasts to be fused are derived from more distantly related species, the genetical background of nutritional complementation will be more complex. Heterokaryon formation is the first event, and the heterokaryotic state can be stable. Synkaryosis is also possible, leading to complicated genetic situations not existing in nature. One can fully agree with Dales and Croft (40) that, instead of the intrinsically homospecific terms "diploid" or "aneuploid", "heteroploid" and "partial heteroploid" should preferably be used in these heterospecific cases.

Such a situation has been observed in the case of the somatic hybrids of *Penicillium roquefortii* and *P. chrysogenum* (41). After protoplast fusion of auxotrophic mutants slowly growing prototrophic colonies developed which could be classified into three types. Type 1 colonies were morphologically normal, grew faster on nutritionally rich media than on minimal medium and produced selectively auxotrophic *P. roquefortii* conidia on rich media. Type 2 colonies were morphologically aberrant in consisting of a loosely meshed network of broadly spreading hyphae. They also produced selectively auxotrophic *P. roquefortii* conidia on rich media. In contrast to these, type 3 colonies sporulated on minimal medium, released large prototrophic conidia, and were similar to *P. chrysogenum* in morphology. All types produced penicillins of the same chemical composition as those of *P. chrysogenum*. Type 1 and 2 colonies were heterokaryotic, whereas type 3 might have been either full heteroploid or partial heteroploid.

Interspecific hybrids of auxotrophic mutants of *P. citrinum* and *P. cyaneo-fulvum* (42) produced slowly growing colonies on minimal medium and conidia of both species were released. More vigorously developing sectors appeared in these colonies with white, large and stable prototrophic conidia which were assumed to contain the complete set of chromosomes of both the complementing species. Markers of both species in almost equal numbers were found on induced segregation. Among the segregants partial heteroploids could also be detected.

No details are known about the genetic background of interspecific complementation after protoplast fusion of auxotrophic strains of *Candida tropicalis* and *Saccharomycopsis fibuligera* (43). Cells of the prototrophic fusion products proved uninucleate and exhibited assimilation spectra resembling those of *Candida* or *Saccharomycopsis* or both. The hybrids were, perhaps, partial heteroploids.

Partial Heteroploid Formation (Bidirectional)

If protoplasts of auxotrophic cells of the distantly related species of *Aspergillus nidulans* and A. *fumigatus* were fused, slowly growing abnormal colonies were produced with a characteristically low frequency (44, 45); the interspecific fusion frequency was at least 5 orders of magnitude less than that of the intraspecific one in either species. The heterokaryotic or full heteroploid states were so transitory that heterokaryons or full heteroploids ("interspecific diploids") could not be isolated. Presumably, both the heterokaryotic and the diploid states are lethal. The heteroploid state may exist only in a partial form where the complemented cells harbour the complete genome of one of the partners and only one or a few chromosomes from the other. On minimal medium the complemented colonies could be maintained indefinitely by transferring either mycelia or conidia. On nutritionally rich medium the complemented cells rapidly segregated one of the partners. The segregation was bidirectional, since either the parental A. *nidulans* or A. *fumigatus*, but never both, could be recovered.

Partial Heteroploid Formation (Unidirectional)

Protoplasts of stable monoauxotrophic strains of *Kluyveromyces lactis* and *K. fragilis* were successfully fused (46). The fusion colonies were able to maintain their prototrophy even in complete medium. In general, the fusion cells were larger and contained more DNA than those of either of the parent species. On the other hand, in most cases the DNA content was lower than the combined DNA content of the two parent implying that subsequently to fusion loss of chromosomes had occurred. At the same time, a selective retention of *K. fragilis* and loss of *K. lactis* mitochondrial DNAs was observed.

CONCLUDING REMARKS

The rich diversity in the genetic background of nutritional complementation after intraspecific and interspecific fungal protoplast fusion is remarkable. In fact, this genetic spectrum is considerably wider than that found in natural sexual or parasexual processes. Results of recent experiments with different *Candida, Schizosaccharomyces, Aspergillus, Penicillium* and *Trichoderma* species show that yet further variations of nutritional complementation may be described (in preparation). New variations are also expected with other species (47). These new possibilities together with the fact that in many cases protoplast fusion is the only known procedure for combining different genomes, clearly demonstrate the importance of induced protoplast fusion in both basic and applied fields of fungal research.

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PROTOPLAST FUSION - A NEW APPROACH TO INTER-SPECIES GENETIC MANIPULATION AND BREEDING IN FUNGI

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The parasexual cycle in filamentous fungi provides a mechanism for genetic modification in some of the many species that lack sexual reproduction. As a rare event, a somatic heterozygous diploid nucleus can arise through the spontaneous fusion of two genetically different nuclei in the mycelium of a heterokaryon. During subsequent divisions chromosomes of the diploid nuclei may undergo mitotic recombination and re-assortment ultimately giving rise to new haploid nuclei, by loss of chromosomes, which carry new gene combinations arising from the interaction of the two genomes (1). The developments in recent years involving the isolation, culture and fusion of fungal protoplasts have added a new dimension to these parasexual phenomena (2). Firstly the method allows the relatively easy synthesis of heterokaryons and this has proved important and valuable in fungi where the formation of heterokaryons by hyphal anastomoses is difficult (3), and secondly through inter-species fusions the interaction of genomes from two different species has opened up a whole new area of fungal genetics and molecular biology. Many of the developments in this area of protoplast research stem from two reports at the previous Symposium in this series held in 1975 (4,5) and the aim of this contribution is to review this work in a comparative manner with the inclusion of some unpublished observations, as well as an assessment of protoplast fusion as a tool in fungal breeding.

Abbreviations for genetic markers
ad(adenine), arg(arginine), bi(biotin), cho(choline), his(histidine), lac(lactose), lys(lysine), met(methionine), nic(nicotinic acid), phen(phenylalanine), pro(proline), pyro(pyrodoxine), ribo(riboflavin), w(white spores) acr(acriflavine resistance), ben(benlate resistance), can(canesten resistance).

PROTOPLAST FUSION AND THE SELECTION OF FUSION PRODUCTS

The induced fusion of fungal protoplasts using polyethylene glycols (PEG) as fusogens is currently the universally accepted procedure in all laboratories engaged in this work. Despite its toxicity (6) PEG is a more efficient fusogen in comparison to others that have been used (7,8). Slight variations in the fusion protocol have been adopted in different laboratories and details of some of these have been collected together in a recently published practical handbook (9).

The selection of fusion products is based upon nutritional complementation that occurs in heterokaryotic protoplasts derived from two suitably selected auxotrophic strains. Selection of the reverting protoplasts on a minimal medium is therefore strong. Ideally auxotrophic strains with several requirements should be used as the parental strains although controls for back mutation are easily set up. This strong selection using a minimal medium has not proved effective in every case. Several of the crosses prepared between strains of Cephalosporium acremonium failed to give colonies when the fusion mixture was plated on minimal medium, but plating on a variety of selective media gave a range of recombinant progeny (3).

The degree of fusion between any two strains is expressed as the fusion frequency or complementation frequency and is derived as the ratio :

$$\frac{\text{colonies developing on minimal medium}}{\text{colonies developing on complete medium}}$$

The values obtained for fusion frequencies in different crosses are very variable and the differences arise from the fact that aggregates of protoplasts that remain after PEG treatment are probably very irregular in size.

The heterokaryotic colonies that develop following protoplast fusion are generally very weakly growing and irregular in form (10. Figure 1.). Evidence for the heterokaryotic nature is generally based on the segregation of the two parental strains that occurs when they are grown on complete medium.

Methods of selection of fused protoplasts first used with animal cells, plant protoplasts and bacterial protoplasts have been attempted with a variety of fungi (Hamlyn, Smith and Peberdy, unpublished data). In the first of these inhibitors are used that cause irreversible blocks in key metabolic pathways (11) which are compensated in the fusion product allowing its development. The concentrations and exposure times used in the animal (11) and the plant protoplast(12) experiments have proved inadequate with fungi. This method is clearly very time-saving because it obviates

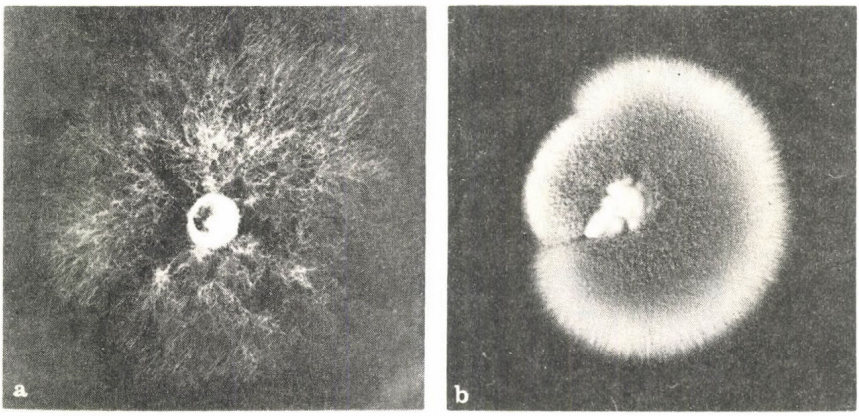

Fig. 1. Interspecific heterokaryon obtained by fusion of protoplasts of Aspergillus nidulans and A. rugulosus growing on (a) minimal medium and (b) on complete medium. The segregation of the two parental types is seen on complete medium. (From Kevei and Peberdy, 10).

the need to produce mutant strains. Another interesting approach has been devised by Fodor, Demiri and Alföldi (13) for the fusion of Bacillus megaterium protoplasts. In this technique protoplasts of one strain are heat treated prior to fusion and those of the other strain function as recipients for markers from the non-viable protoplasts. The procedure provides a means of counterselection when few markers are available and when a prototrophic parent is used. Unfortunately the method could not be applied effectively to fungi because of problems of survival of spores and hyphal fragments in the protoplast suspension of the heat treated strain (Hamlyn, unpublished data). Selection methods in which prototrophic strains can be used are of considerable interest particularly in the application of fusion techniques to commercial strains where there is a reluctance to introduce selective markers by mutagenic procedures.

DEVELOPMENT AND NATURE OF INTERSPECIFIC HYBRIDS

The interest in fusion of protoplasts from taxonomically distinct species stems from the development of relatively stable forms from the heterokaryon. In both Aspergillus (10) and Penicillium (14,15) crosses, hybrids arose as vigorously growing sectors or as complete colonies from heterokaryons following subculture from the protoplast culture plates to non-stabilised minimal medium. An important property of these hybrids is their stability on complete medium; in an Aspergillus nidulans x rugulosus hybrid stability was

maintained by hyphal transfers because segregation occurred at sporulation. In contrast the Penicillium hybrids gave no segregation at sporulation (14,15; Figure 2). Other features of the hybrids are the extensive secretion of a brown pigment as well as colony pigmentation, in the Aspergillus, and the total absence of colony pigments in the Penicillium hybrids despite the use of strains with pigmented spores in their production.

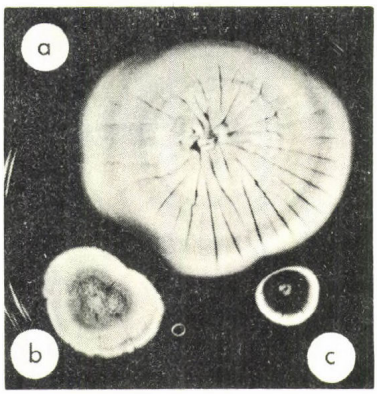

Fig. 2. Hybrid derived by protoplast fusion between Penicillium chrysogenum and P. cyaneo-fulvum. (a) Hybrid colony (b) Penicillium chrysogenum (c) Penicillium cyaneo-fulvum. The colonies were grown on complete medium. (From Peberdy, Eyssen and Anné, 14).

The first attempts at crosses between less related species failed to produce stable progeny of the type just described Following fusion between Aspergillus nidulans and Aspergillus fumigatus (16) and between Penicillium chrysogenum and Penicillium roqueforti (17) colonies were isolated which showed properties typical of aneuploids with the rapid segregation of one of the parental strains when cultured on complete medium. Further investigations on the fusion products obtained from the Penicillium chrysogenum x Penicillium roqueforti cross produced a variety of progeny which could be distinguished by small, but constant, differences in colony morphology and by differences in stability when cultured on complete medium. The occurrence of this multiplicity of types further supports the idea of the progeny of this cross to be aneuploid (Peberdy and Anné, unpublished data). In other experiments involving the distally related Penicillium cyaneo-fulvum and Penicillium citrinum, Anné and Eyssen (15) succeeded in obtaining stable hybrid forms from the fusion products and these resembled

the hybrids derived from Penicillium chrysogenum and Penicillium cyaneo-fulvum (14). Evidence has also been obtained recently for hybridization between Aspergillus niger and Aspergillus rugulosus (El-Mesillati, unpublished data). Several colony types were obtained following transfer of the fusion products onto minimal and complete media. The behaviour of some of the isolates suggested they were aneuploid i.e. they readily segregated the Aspergillus niger parent. Other colonies were more stable and showed evidence of some degree of genetic interaction with the development of non-parental segregants. To allay doubts of causes other than heterokaryosis as the explanation of colony development following protoplast fusion several controls additional to those described in an earlier publication (10) were carried out (Table 1) but in no case was colony development found.

TABLE 1 Controls used in an interspecific cross between Aspergillus niger (arg, his, acr, ben, w) and Aspergillus rugulosus (met, ribo, ad, can)

a) Back Mutation

High density plating of protoplasts (5×10^6 ml^{-1}) of each strain, separately, on minimal medium.

High density plating of conidia (5×10^8 ml^{-1}) of each strain, separately, on minimal medium.

Plating macerated mycelium of each strain, separately, on minimal medium.

b) Influence of nutrient supplementation

PEG treated protoplasts of Aspergillus rugulosus plated at high density (5×10^6 ml^{-1}) on MM supplemented with histidine and arginine.

PEG treated protoplasts of Aspergillus niger plated at high density (5×10^6 ml^{-1}) on MM supplemented with methionine, riboflavin and adenine.

c) Cross-feeding

Mixture of protoplasts from both strains (2.5×10^6 ml^{-1} of each), with PEG treatment, plated on MM

Reciprocal mixtures of protoplasts (5×10^6 ml^{-1}) and macerated hyphae from both strains plated on MM

Reciprocal mixtures of spores (5×10^8 ml^{-1}) and macerated hyphae from both strains plated on MM

The vigorously growing sectors that arise from the heterokaryotic fusion products are assumed to be the result of nuclear fusion events and in some instances subsequent chromosome loss by non-disjunction. The clearest evidence for this view is genetic with the re-assortment of markers

from the two parents into new non-parental combinations (see below). Cytological evidence was obtained from the hybrid obtained between Aspergillus nidulans and Aspergillus rugulosus (10, Table 2) which showed that the hybrid nuclei contained twice the level of DNA as the two parental strains.

TABLE 2 DNA content per nucleus of Aspergillus nidulans and Aspergillus rugulosus and the hybrid obtained by protoplast fusion (From Kevei and Peberdy, 10)

Strain	No. of nuclei per protoplast	DNA content per 10^6 protoplasts (ug)	DNA content per nucleus (ug)	Ratio DNA content Hybrid/ Parent
Aspergillus nidulans	1.50 ± 0.18	7.05	4.70×10^{-6}	2.29
Aspergillus rugulosus	2.16 ± 0.32	11.75	5.43×10^{-6}	1.98
Hybrid	2.79 ± 0.27	30.10	10.78×10^{-6}	

Not all the heterokaryons obtained in the Aspergillus nidulans x Aspergillus rugulosus and the Penicillium chrysogenum x Penicillium cyaneo-fulvum crosses gave hybrid colonies. In the former case the frequency of hybrid development increased with prolonged culture of the heterokaryon under the strong selective pressure of growth on minimal medium. Protoplasts prepared from the Aspergillus heterokaryon gave both parental colony forms and the hybrid when cultured with the incidence of hybrid colonies increasing when older heterokaryons were used to prepare the protoplasts. The frequency of hybrid development in the Penicillium cross was about 60 per cent (Smith, unpublished data).

GENETIC SEGREGATION OF INTERSPECIFIC HYBRIDS

The hybrids derived from Aspergillus nidulans and Aspergillus rugulosus gave segregants both spontaneously and following induction by growth on a haploidizing agent (10, 18) but induction was necessary to produce segregants from the Penicillium hybrid (14). The subsequent behaviour of the segregants derived from these hybrids indicates heterogeneity in their ploidy and in this manner provide a comparable situation to that found in the haploidization of an intra-specific diploid. Many of the segregants from inter-specific hybrids are aneuploids giving second order segregants either spontaneously or following further induction. The segregants of greatest interest are those

that show a re-assortment of the two sets of parental markers. In more recent experiments, in which strains carrying more markers have been used, the incidence of these segregants was found, not surprisingly, to be considerably higher (18, Table 3).

TABLE 3 First order segregants from a hybrid obtained from Aspergillus nidulans (ad, phen, pyro, lys, nic) x Aspergillus rugulosus (pro, ribo)

Strain phenotype (auxotrophy)	Number of segregants
Parentals	
pro, ribo	2
ad, phen, lys, nic, pyro	3
Non-parentals	
ad, nic	1
ad, nic, lys	2
ad, nic, phen, lys	1
ad, ribo, nic, pyro, lys	2
nic	17
nic, phen	4
phen	2
pro, nic	15
pro, ad, lys	1
pro, nic, phen	6
pro, nic, lys	3
pro, ribo, phen, lys	1
pro, ad, nic, pyro, lys	1
pro, ad, ribo, nic, lys	1
pro, ad, ribo, nic, pyro, lys	1
ribo, nic, phen, lys	1

The use of genetically mapped strains such as the master strains of Aspergillus nidulans (19) has provided the opportunity to consider whether the assortment of chromosomes that occurs during the haploidization of the inter-specific hybrids is a random or an ordered process. Analysis of the segregants derived from these crosses has shown that the former situation applies (18, Table 4). The occurrence of mitotic recombination in inter-specific fungal hybrids has not yet been established.

Another property of the inter-specific hybrids and their segregant progeny relates to the behaviour of the parental spore colour markers. The occurrence of green spored segregants from an Aspergillus nidulans x rugulosus hybrid derived from two yellow spored parents and the green spored

second order isolates developing from yellow spored first order segregants of the Penicillium chrysogenum x cyaneofulvum hybrid are examples of the atypical behaviour and interaction of these genes.

TABLE 4 Frequency of Aspergillus nidulans linkage groups in segregants derived from the hybrid between Aspergillus nidulans FGSC 407 and Aspergillus rugulosus R1 (From Kevei and Peberdy, 18)

Aspergillus nidulans linkage group	Frequency in segregants
I	2
III	7
IV	5
V	14
VI	5
VII	7

Parental genotypes and linkage groups

Aspergillus nidulans adE20, biA1 (I); AcrA1 (II); phenA2 (III); pyroA4 (IV); lysB5 (V); lacA1 (VI); choA1 (VII); riboB2 (VIII)

Aspergillus rugulosus pro 1

PROTOPLAST FUSION AND BREEDING

A major interest in protoplast fusion in fungi relates to its application in breeding and strain modification in industrially important organisms. There are likely benefits in the use of the technique in both intra- and inter-specific crosses. At the intra-species level the fusion technique problems of heterokaryon development that have been encountered in some fungi e.g. Cephalosporium acremonium (3) when the conventional technique using hyphal anastomses is practised. At the inter-species level a number of developments are possible for the future. In the Penicillia hybridization of Penicillium chrysogenum with other sexually reproducing Penicillium species (Smith, unpublished data) could yield recombinants with both the properties of antibiotic production and sexual reproduction. The latter property could then be further exploited in a conventional breeding programme. Other benefits of inter-species breeding could be the introduction of the capability to utilise a broader range of growth substrates into commercial strains, modification of existing products and the

possibility of novel products resulting from the interaction of metabolic pathways controlled by the two parental genomes.

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PROTOPLAST FUSION AND THE GENETICAL ANALYSIS OF VEGETATIVE INCOMPATIBILITY IN ASPERGILLUS NIDULANS

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In the last four years the use of polyethylene glycol (PEG), to induce fusion between protoplasts derived from different, though often closely related, filamentous fungal species, has facilitated the isolation of regenerated mycelial material of genetically hybrid nature (1 - 7). From interspecific fusions of closely related species (1, 5, 6, 7), as with intraspecific fusions (1, 2, 8, 9, 10), true balanced heterokaryons have been isolated under nutritional selection, from which both parental strains can be recovered. These interspecific heterokaryons, though, display characteristics, such as drastically reduced conidiation, slow growth and increased secretion of pigments, which indicate a deleterious somatic interaction between the component homokaryons. Subsequent hybrid strains have been obtained (5, 6, 7), which are presumably the result of nuclear fusion events occurring within the heterokaryons, and these, too, have impaired growth forms. From evidence, such as the ratio of DNA content to the number of nuclei within protoplast samples obtained from the hybrid (5) and upon limited genetic information obtained from the analysis of spontaneous and induced segregants from hybrid strains (5, 6, 7), certain inferences as to the genetical composition of the hybrids have been drawn, and it has been suggested that certain hybrid strains may be diploid (5, 6, 7). It must be speculative to apply this intrinsically homospecific term to situations in which the haploid chromosome complements of both parent strains are not known, either from genetical analysis or from cytological evidence. It may be, then, that both parental chromosome complements are not fully represented within the hybrid nuclei, or if they are, they may well not be equal and are unlikely to be fully homologous. 'Partial heteroploids' or 'possible heteroploids' may well be more suitable descriptions of these hybrid situations.

Genetically, the most thoroughly studied examples to date have been three hybrids isolated from strains of Aspergillus rugulosus, bearing single auxotrophic requirements, and master strains of A. nidulans which are marked on all eight linkage groups (7). Analysis of benomyl and chloral hydrate induced progeny samples from the hybrids has revealed the segregation of most, or all, of the markers possessed by the haploid parents. This indicates that both the original markers and the complementing wild-type genes from the respective parents have been retained in the hybrid and therefore that the hybrid is heterozygous, at

least for the markers which show phenotypic segregation. It must be stressed that the markers and the corresponding wild-type genes may not necessarily be allelic between the two species and the loci involved are even less likely to have the same linkage relationships. This, compounded with the sampling procedure employed, may go some way towards explaining the surprising segregation patterns obtained.

In situations where more distantly related species have been fused (2, 3, 4), balanced heterokaryons have not been isolated. However, nutritionally complementing hybrid strains have been recovered directly from platings of the fusion mixture on minimal or selective media. These hybrids are again severely restricted and morphologically abnormal when compared to intraspecific fusion products of either of the parental species. From analysis of segregants obtained from the hybrids it would seem that the hybrids themselves are interspecific aneuploids, though partial heteroploids may be a preferable description. The majority of the genetical information appears to be provided by one parent in association with a much smaller contribution from the other. It would appear that the preferential loss of chromosomes from one species has occurred with just those genetic elements necessary for nutritional complementation being retained.

All of the interspecific hybridization experiments so far reported have revealed that these situations are highly unstable genetically and that they are severely affected morphologically when maintained by strong nutritional selection. This could well be attributable to chromosome structural variation between the species, deleterious allelic or non-allelic interactions due to the poorly co-adapted nature of the combined nuclear genomes, and deleterious interactions between organelle genetic elements and the associated poorly co-adapted nuclear genomes.

One such mechanism which may operate interspecifically, can be detected operating within many fungal species and this is heterokaryon or vegetative incompatibility. A heterokaryon incompatibility system has been observed in the Ascomycete, A. nidulans (see ref.11 for review) and here independent wild isolates have been grouped according to their ability to form heterokaryons. Members of any one heterokaryon compatible (h-c) group all form heterokaryons with each other, but do not normally do so with members of any other group. Under normal circumstances this inability to form heterokaryons precludes any further stages of parasexual development. However, it has proved possible to sexually cross strains belonging to different h-c groups and subsequent progeny analysis has revealed that this incompatibility system is controlled by a number of nuclear genes, the het genes, and that allelic differences between strains at any one, or more, of these het loci will result in heterokaryon incompatibility. More complex sexual crossing programmes, often involving multiple backcrosses, have enabled the exact number of het gene differences between strains to be ascertained. In this way the number of het genes segregating in crosses between members of h-c groups A and B has been found to be 7, and that these groups differ from the standard laboratory, NRRL 194 derivative 'Glasgow' strains (h-c G1) at 6 and 2 het loci respectively. Consideration of this triangular situation has indicated that 3 different alleles must be operating at one het locus within these 3 h-c groups for this compatibility relationship to be upheld. The two het genes which segregate in crosses between strains of h-c B and h-c G1 have been designated hetA and hetB, with Glasgow strains being designated

hetA1; hetB1, and h-c B strains being hetA2; hetB2. These two het loci were found to be unlinked, the recombinant classes hetA1; hetB2 and hetA2; hetB1 being recovered in equal frequencies with the parental classes from sexual crosses.

The amount of further genetical information that can be gained from the analysis of sexual crosses is limited, mainly by the high meiotic recombination frequency exhibited by A. nidulans and also by the low number of genetical markers at present available within the h-c groups, with the single exception of the Glasgow group of strains. Consequently the possibility of detecting linkage of het genes with other standard genetical markers is remote. Furthermore, as the sexual crossing mechanism is apparently unaffected by parental strains bearing different het gene combinations (12), then this, in itself, precludes investigation of the interaction of differing alleles at individual het loci. This leaves the enigma that the most desirable method for studying the genetical control of the heterokaryon incompatibility system in A. nidulans would be by the observation of heterokaryon formation and by parasexual analysis of subsequent diploid or aneuploid strains derived from heterokaryon incompatible parents, though, under normal circumstances this approach is prevented by the incompatibility system itself. Initial experiments using a protoplast fusion technique, however, indicated that it was possible to isolate heterokaryons and probably diploids from vegetatively incompatible parents (13) and this opened the way for conducting routine parasexual analyses of between h-c differences.

The methods by which protoplasts were liberated from strains of A. nidulans and induced to fuse have been reported in detail elsewhere (14). They are largely derived from the methods used by Peberdy (15, 16) and Ferenczy (17, 18).

To perform a standard parasexual analysis starting with strains of different heterokaryon compatibility, it would have been desirable to have the parental strains marked on all linkage groups. Fully marked master strains are not available at present in any of the h-c groups derived from the Birmingham collection of wild isolates and the amount of work that would be necessary to synthesize such strains made this approach impractical. Master strains already were available in the Glasgow stocks and it was decided to use these in conjunction with strains of different heterokaryon compatibility bearing single auxotrophic requirements, necessary for the nutritional selection of the products of protoplast fusion. This would invoke the assumption that wild-type genes possessed by the non-Glasgow parent were allelic with, and of the same linkage relationships as the markers borne by the Glasgow master strains. It was hoped that the segregation patterns and 'allelic' ratios obtained from the parasexual analyses would indicate whether this assumption was valid.

Protoplast fusion of strain MSD with strain JC.9-8

These two strains are of Glasgow and h-c B compatibility respectively and possess the following genotypes for known, standard markers:

MSD (h-c Glasgow)

suA1	+	yA2	adE20	AcrA1	phenA2	pyroA4	lysB5	sB3	nicB8	riboB2
	I			II	III	IV	V	VI	VII	VIII
+	paba-1.3	+	+	AcrA1	+	+	+	+	+	+

JC.9-8 (h-c B)

Strain JC.9-8 is one of the progeny from a sexual cross between strain MSD and a Birmingham h-c B isolate in which a p-aminobenzoic acid requirement paba-1.3, had been induced. Unfortunately, both strain MSD and JC.9-8 possess the acriflavine resistance marker, AcrA1, on linkage group II, so there was no test for het gene location on that particular chromosome. Protoplast fusion resulted in the isolation, on osmotically stabilized Cz agar, of colonies of thin mycelial morphology, showing the typical uneven growth pattern of balanced heterokaryons. Neither protoplasts from the individual parental strains nor osmotically imbalanced fused protoplasts regenerated on stablilized Cz agar. The colonies that regenerated on the fusion plates developed an intense pink pigmentation in the mycelium which diffused into the surrounding agar. The frequency of spore head formation was low and these were mainly small and unpigmented. In some cases the pink pigmentation within the hyphae had suffused the conidiophore and the whole structure, including conidia, displayed this pink colouration. However, in some spore heads yellow or green conidial pigmentation was observed and yellow-green striped heterokaryotic heads were seen occasionally. The colonies were easily subcultured onto clean, unstabilized Cz agar where the growth retained the pink pigmentation and the very low production of conidia. Breakdown of these colonies on fully supplemented media led to the recovery of parental genotypes only, both for markers and compatibility type, from a sample of 32 purified sectors. The colonies which resulted from protoplast fusion were therefore considered to be heterokaryons formed across a heterokaryon incompatibility group barrier produced by 2 het genes.

Despite the low numbers of conidia produced by the heterokaryons, conidial suspensions could be obtained. Platings of such suspensions in soft agar layers on Cz agar led to the recovery of prototrophic, symmetrically round colonies, characteristic of normal diploid strains, at a frequency of 1.73×10^{-6} of colony forming units plated. These were again thin mycelial colonies displaying a pink pigmentation which was cell-localised and not secreted into the medium. Sporulation was sparse with a small number of unpigmented or pink pigmented conidiophores. Towards the periphery of the colonies sporulation was slightly improved and small, diffuse spore heads of light green conidial pigmentation were observed. These conidia were found to be on average 1.33 x the diameter of normal haploid conidia. Rare yellow-sporing heads were observed within the colonies and occasional sectors of haploid morphology which were usually highly sexual. At this stage these pink colonies were presumed to be diploid. Occasionally, on prolonged incubation, or following mycelial subculture, sectors of normal diploid morphology appeared and these will be discussed later. The pink, presumed diploid colonies were induced to haploidize on fully supplemented media containing benomyl (19, 20). A progeny sample of 97 sectors was collected and analysed for genotype. A summary of the results of this parasexual analysis are given in table 1.

Table 1: Percent recombinants between hetA and hetB and parental markers

	I		III	IV	V	VI	VII	VIII	
	paba	yA	phenA	pyroA	lysB	sB	nicB	riboB	hetB
hetA	53.6	53.6	49.5	51.5	1.0	53.6	62.9	56.7	52.5
hetB	42.3	42.3	56.7	46.4	53.6	1.0	45.4	49.5	

It can be seen from table 1 that the het genes segregated independently from each other and this confirms the data obtained from sexual crosses that these genes are unlinked. The het genes also demonstrated random segregation with the other markers tested, except that hetA shows mitotic linkage with the lysB locus on linkage group V and similarly hetB is mitotically linked with the sB locus on linkage group VI. The allelic ratios obtained in this experiemnt were all approximately 1:1 and this random assortment is indicative of a translocation-free relationship (21, 22) between strains MSD and JC.9-8.

Protoplast fusion of strains bearing different alleles at the hetA locus
In these experiments the recombinant compatibility classes of h-c B x h-c G1 crosses were fused with the parental types in the following combinations:
hetA1; hetB1 (h-c G1) + hetA2; hetB1 (Recombinant)
hetA2; hetB2 (h-c B) + hetA1; hetB2 (Recombinant)

In both cases protoplast fusion, of strains also bearing complementing auxotrophic requirements, led to the recovery on Cz agar of colonies with a sectoring growth pattern, bearing green and yellow spore heads and occasional striped heterokaryotic heads. The removal of nutritional selection caused the breakdown of these colonies and only parental genotypes could be reisolated. The fusion products of the two combinations were virtually identical and any slight visible differences could be accounted for in terms of the plasticity of heterokaryon balance (23). In comparison to heterokaryons produced from compatible pairings there was a slight reduction in spore head size and in sporulation density with aerial hypae and non-conidiating areas of mycelium visible, though no other morphological differences could be observed. Nevertheless, allelic differences for the hetA gene have always been fully effective in preventing heterokaryosis when using standard methods for the production of heterokaryons. Platings of conidial suspensions for diploid recovery led to the isolation of symmetrically round colonies, which were virtually identical in the two combinations. Slightly diffuse spore heads of pale green pigmentation were produced with conidial diameters typically larger than those of haploid conidia and occasionally yellow heads were observed. There was, though, a slight reduction in spore head density when comparison was drawn with standard diploid strains and this slight 'reduction in vigour' enabled the visual identification of sectors of apparently normal diploid morphology. In a situation where parasexual mapping was feasible (strain MSD - hetA1; hetB1 + h-c B strain - hetA2; hetB1) the results confirmed the location of hetA on chromosome V.

Protoplast fusion of strains bearing different alleles at the hetB locus.
Here the following compatibility combinations were employed:
hetA1; hetB1 (h-c G1) + hetA1; hetB2 (Recombinant)
hetA2; hetB2 (h-c B) + hetA2; hetB1 (Recombinant)

Heterokaryons and diploid strains obtained following protplast fusion in these experiments were very similar morphologically to those of the MSD + JC.9-8 fusion experiment. They displayed many of the characteristics seen in heterokaryons and diploids from compatible pairings except for the intense pink pigmentation to the thin mycelium and the drastically impaired sporulation. It would appear that much of the morphological abnormality observed in the products of h-c G1 with h-c B protoplast fusions can be directly attributed to allelic differences at the hetB gene. Parasexual analysis of the diploids heterozygous for hetB confirmed the map location of this gene on chromosome VI and in the diploid colonies themselves, sectors of apparently normal diploid morphology were again observed.

Analysis of the normal morphology diploid sectors

Haploidization of a sample of these sectors revealed that they were all recombinant diploids. Recombination had taken place in such a way as to render homozygous, linkage groups which initially possessed heterozygous alleles for het genes. For example the normal morphology diploid sectors emanating from the MSD/JC.9-8 diploid were found to be homyzygous for both linkage groups V and VI, while heterozygosity was retained at the remaining 6 chromosomes. It would seem reasonable to assume that these recombinant diploid strains were selected in direct response to het gene heterozygosity within the impaired morphology, heterozygous diploid colonies, i.e. there was a strong selection pressure favouring het gene homozygosity. Using this as an initial premise, two models can be proposed of mechanisms which would achieve het gene homozygosity (14, 24, 25). Firstly, by mitotic recombination (Fig.1) and secondly, by non-disjunctional segregation of whole chromosomes involving transient trisomy (Fig.2). At present there is no evidence to favour either of these models, though experiments are in progress, using tester strains multiply marked on linkage groups V and VI in order to elucidate the underlying mechanisms of this genetic instability.

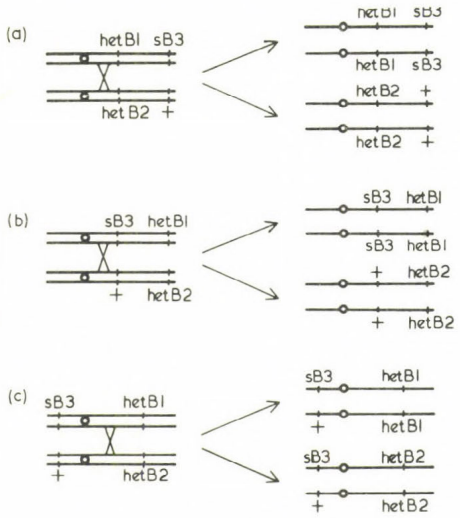

Fig.1: A model for achieving het gene homozygosity for linkage group VI via mitotic recombination. The result indicated in (c) has never been recovered.

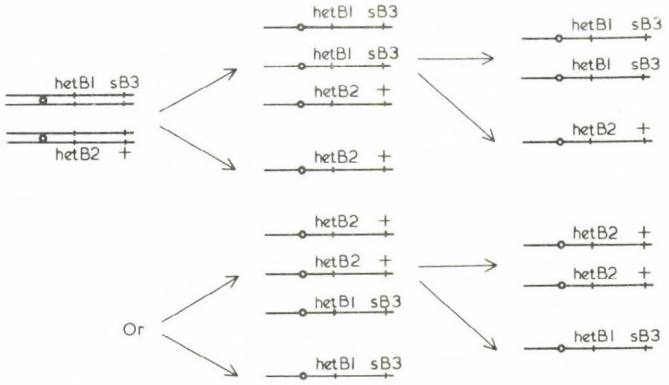

Fig.2: A model for achieving het gene homozygosity for linkage group VI via non-disjunction.

Protoplast fusion of strain MSD with strain 65-4.

Strain 65-4 is a member of h-c A and is a mutant derivative, possessing a requirement for p-aminobenzoic acid, paba-65.1 , of wild isolate 65 of the Birmingham strain collection. Glasgow strains differ from strains of h-c A at 6 het loci (24) and this number of het gene differences would make a parasexual analysis extremely complicated. Heterokaryon compatibility testing is routinely performed by inoculating strains of different spore colour together, without nutritional selection and investigating for the presence or absence of heterokaryotic conidial heads. The presence of these striped heads is indicative of a neutral or non-selected heterokaryon. This method is very useful when tester strains of known compatibility are available as was possible with the h-c B: h-c G1 compatibility difference. A cross performed between strains of h-c A and h-c G1 would be expected to liberate 64 compatibility classes among the progeny. Strains of both green and yellow spore colour would be required for each class and therefore 128 compatibility tester strains would be needed if a full parasexual analysis was attempted.

However, parasexual haploidisation analysis involves the reassortment of complete, unrecombined, linkage groups. Mitotic crossing-over events usually only affect this situation in less than 1% of cases. Consequently, in a haploidisation analysis, if all 8 linkage groupswere monitored with standard markers then it would be generally expected that het alleles would segregate with near complete linkage to the alleles of standard markers located on the same linkage group (26). It should therefore prove possible to analyse a parasexual progeny sample among its constituents. For example, take the 2 following hypothetical progeny strains of an h-c G1 with h-c A parasexual cross:

Linkage Group	I	II	III	IV	V	VI	VII	VIII
Strain 1	+ y	Acr	+	+	+	s	nic	ribo
Strain 2	paba +	Acr	+	+	+	s	nic	ribo

———— = Linkage groups derived from Glasgow parent
------- = Linkage groups derived from h-c A parent

If these two strains were compatible it would indicate that there was no het gene location on linkage group I. Then by pairing further progeny strains heterozygous for linkage group I, which is necessary for the spore colour difference, and for each other linkage group in turn then tests can be made for the location of het genes on the remaining 7 chromosomes. For example, the following two progeny strains would test for location on chromosome VII:

Linkage Group	I	II	III	IV	V	VI	VII	VIII
Strain 3	+ y	+	phen	pyro	lys	+	nic	ribo
Strain 4	paba +	+	phen	pyro	lys	+	+	ribo

Protoplast fusion of strain MSD with strain 65-4 led to the regeneration of small colony centres composed of just a few hyphae. Only 5 of these colony centres regenerated further to display a thin, non-conidiating, but sectoring mode of growth characteristic of balanced heterokaryons. In the absence of nutritional selection both parental types were recovered. Suspensions of mixed mycelial fragments and conidia were made from these heterokaryons and soft-agar layer platings enabled the recovery of symmetrically round colonies with a thin mycelium and very few conidia. These colonies were assumed to be diploid and haploid progeny samples were collected. Analysis of the standard markers revealed that all markers were segregating independently from their respective wild-type alleles, except for the sB marker on linkage group VI. All the 256 haploid progeny segregants were wild-type with respect to this marker. This unfortunately meant that a chromosome substitution test for location of het genes on chromosome VI was not possible. The tests for the remaining 7 linkage groups were possible and the results indicated that het gene differences between h-c G1 and h-c A were located on linkage groups II, III, V and VII (Table 2).

Table 2: The location of het gene differences between h-c G1 and h-c A.

Linkage Group	I	II	III	IV	V	VI	VII	VIII
Presence(+) or Absence(-) of het gene differences.	-	+	+	-	+	?	+	-

Protoplast fusion of strain MSD with strains 106-2 and 43-5

Strains 106-2 and 43-5 both possess induced requirements for p-aminobenzoic acid and are members of h-c groups Q and E respectively. No information was available regarding the number of het gene differences between these groups and Glasgow strains so it was decided to use the chromosome assay technique as described for the h-c A/h-c G1 fusion.

In both cases protoplast fusion led directly to the recovery of colonies stable on complete medium, though a single heterokaryotic colony was recovered from the Q/G1 fusion which proved impossible to subculture. The more stable colonies all demonstrated drastically impaired growth being mycelial, aconidial forms with much of the hyphal extension being through

the agar. The Q/G1 colonies were pinkish-orange in pigmentation whereas with E/G1 two morphological types were recovered, one red and the other pigmentless (white). The red colonies were often observed to sector out areas of the white mycelial morphology. Marker segregations among parasexual progeny samples obtained from all three of these morphological types indicated that the red colonies (E/G1) were fully heterozygous diploids while the white (E/G1) and pinkish-orange (Q/G1) colonies were recombinant diploids being homozygous for linkage group VI. Chromosome substitution compatibility tests were performed using these progeny samples and suitable progeny strains were further selected in order to set up sexual crosses. The number of compatibility classes present amongst the progeny of such crosses would then reveal how many het gene differences were operative on each linkage group. A summary of these results is presented in Table 3.

Table 3: The number and location of het genes heterozygous within Q/G1 and E/G1 diploid strains.

Linkage Group			I	II	III	IV	V	VI	VII	VIII
Q/G1	Presence(+)or Absence(-)of het gene differences		-	-	+	-	+	?	+	-
Number of het gene differences			0	0	2	0	1	1	1	0
E/G1	Presence(+)or Absence(-) of het gene differences	Red diploid	-	-	+	-	+	+	-	-
		White diploid	-	-	+	-	+	?	-	-
Number of het gene differences			0	0	2	0	2or3	1	0	0

It can be seen from Table 3 that sexual crossing data confirmed the presence of a single het gene difference on linkage group VI in both cases. It would seem that homozygosity for this chromosome within the Q/G1 diploid and the white E/G1 diploid was selected in response to a strong selection pressure imparted by heterozygosity of that linkage group, possibly attributable to allelic differences at the het loci. The mechanisms by which this could take place could well be mitotic recombination or whole chromosome non-disjunction as proposed for the normal morphology sectors of the B/G1 diploid. Drawing inference from these results, the recombinant nature of the A/G1 diploid might well indicate the presence of het gene activity on linkage group VI, but this has yet to be shown. Work is currently in progress to continue the study of the A/G1, Q/G1 and E/G1 het gene differences. As a result of sexual crosses between selected parasexual progeny a collection of strains is being built up, all of which differ from Glasgow strains at single het loci. These strains will be useful for the investigation of the interactions caused by single het gene allelic differences, and for further genetical analysis and also for possible characterisation of het gene products.

Protoplast fusion of an A.nidulans Glasgow strain with a strain of A. quadrilineatus.

These two species belong to the A. nidulans group of species (27). It

would be of interest to investigate whether the procedures described here for studying the heterokaryon incompatibility system could be applied to an interspecific situation. Firstly, it was necessary to ascertain whether heterokaryons or nuclear fusion heteroploids could be obtained between these two species and the two strains employed for this initial fusion were only marked with single auxotrophic requirements:

A.nidulans (G1):- pabaA6. A. quadrilineatus:- y-12.1 pyro-12.1

Colonies appeared on the regeneration plates, which were thin mycelial, aconidial, sectoring growth colonies secreting an intense red pigmentation. Both parental types could be recovered from these colonies in the absence of nutritional selection, so they would appear to be balanced heterokaryons formed between the two species from which partial or fully heteroploid strains could possibly be obtained. In this context one further colony type, which was obtained from the regeneration plates, bore a very striking resemblance to chromosome I disomics of A. nidulans (28). Areas of normal haploid A. nidulans morphology of both green and yellow conidial colour sectored out from these colony centres . The yellow-sporing sectors were all found to be pyridoxine requiring and the green sectors were all p-aminobenzoic acid requiring. Furthermore the two types of sector were heterokaryon compatible with each other forming striped heads readily indicating that no het genes differences were operating interspecifically for chromosome I. These results suggest that the colony was a chromosome I disomic and it may well imply that there is a great deal of structural homology for this particular chromosome between the two species. However the possibility of recombinational events such as mitotic recombination occurring prior to the formation of this partial heteroploid colony cannot be excluded. Nevertheless, it may well prove possible to investigate interspecific chromosomal relationships and interactions by the direct selection of particular disomic strains.

SUMMARY

The investigations described here have indicated successful methods for studying the heterokaryon incompatibility system operating within wild populations of A. nidulans. These have enabled het genes to be ascribed to linkage groups following parasexual analysis of B/G1, A/G1, Q/G1 and E/G1 inter-h-c group diploids and, in the Q/G1 and E/G1 situations, the number of het gene differences per chromosome has been ascertained by sexual analysis. These methods combined possibly with that of selecting aneuploid, or partial heteroploid, strains may well be of use for studying the regeneration products of interspecific protoplast fusions. However, problems of genome instability have regularly been encountered within the inter-h-c group diploid strains of A. nidulans and reactions of this sort are likely to be significant in interspecific situations.

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THE TRANSFER OF MITOCHONDRIA BETWEEN SPECIES OF ASPERGILLUS

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INTRODUCTION

In fungi, the transfer of a genetically controlled character from its association with one nuclear background to a second nuclear background following the intervening step of heterokaryosis has long been considered as evidence for the extranuclear location of the genetic determinant of that character (1,2). The recent rapid development of the study of organelle genetics has resulted in many examples of extranuclearly inherited characters for which there is strong evidence that their genetic determinants are located on the mitochondrial (mit) DNA. The transfer of these characters in heterokaryons or in experiments which achieve essentially the same result (e.g. in sexually produced strains where the character shows maternal inheritance, or in diploid clones arising from zygotes as in yeast) has been demonstrated (see ref. 3 for general review).

The recombination of mitochondrial genetic markers was first shown to occur in yeast more than ten years ago (4) and since then it has been demonstrated in Aspergillus nidulans (5) and in Podospora anserina (6). In yeast a considerable amount of information is now available and a general model for the recombination and segregation of mitochondrial genes has been published (7). Genetic recombination has been shown to involve the physical recombination of mitDNA (8). The mitochondrial genome of yeast is now thought to consist of a number of relatively G + C rich genes and regulatory sequences, which make up about 50% of the total genome, and very A + T rich internally repetitive spacer sequences (9,10). Differences in the physical structure of the mitDNA of different wild type strains has been reported (11,12) and, using restriction endonuclease fragment analysis, the frequent occurrence of non-parental restriction fragments in the mitDNA of the progeny of a cross between two such wild type strains indicates the high frequency with which mitochondrial recombination takes place (13). The strain specific differences in the mitDNA appear to be due to insertions or deletions in the A + T rich spacers and it has been suggested that the new restriction endonuclease fragments arise as a result of unequal crossing over in these spacers (11,13). In addition, recombination resulting from a process of gene conversion at sites involving insertions and deletions has been proposed for the ω and the var1 loci (7, 14, 15, 16).

Though our understanding of the mitochondrial genetic system of yeast

greatly exceeds that of any other species, considerable advances in the study of mitochondrial genetics in Aspergillus nidulans have been made. Three phenotypic classes of extranuclear mutations have been studied: oligomycin resistance (17), chloramphenicol resistance (18) and cold sensitivity (19). A fourth class, partial suppression of cold sensitivity, has been described recently (20). The genetic linkage of these mutations (20, 21, 22) and their effects on mitochondrial functions indicate that all are located on a single extranuclear genetic element, presumably the mitDNA.

A physical map of A. nidulans mitDNA has been produced by the analysis of restriction endonuclease fragments (23). The fragment patterns produced by restriction enzymes, for example HindIII, HaeIII, and EcoRI, from the mitDNA of three independent genetically unrelated isolates of A. nidulans, that is one from each of three heterokaryon compatibility groups (24), are indistinguishable from each other, suggesting that the mitDNA of this species is structurally homogenous (Turner, unpublished results). This should be confirmed by the use of other enzymes and the analysis of other strains. However, when a similar analysis is carried out on the mitDNA of the distinct but taxonomically related species A. quadrilineatus and A. nidulans var. echinulatus (25), differences in the pattern of restriction fragments are then seen (Turner, Earl and Lazarus, unpublished results). It would be of interest for studies of mitochondrial recombination, mitochondrial genome organisation, nuclear-mitochondrial interactions and mitochondrial genome evolution to be able to produce heteroplasmons between these species, thus allowing the different mitochondrial genomes and nuclear backgrounds to interact.

Direct transfer of mitochondria by microinjection has been carried out in Neurospora crassa (26) but it is unlikely that this technique would be of any general applicability in Aspergillus. Also, in Aspergillus, the transfer of cytoplasmic markers from one strain to another by way of heterokaryosis is prevented or severely restricted by the heterokaryon incompatibility system (27). In the yeasts, Saccharomyces cerevisiae and Kluyveromyces lactis, the transfer of mitochondria from one strain to another by protoplast fusion (28, 29) and the direct fusion of isolated mitochondria with protoplasts in S. cerevisiae (30) have recently been reported. In this paper the transfer of mitochondrial genes between heterokaryon incompatible strains of A. nidulans and between different species of Aspergillus by the use of protoplast fusion is described. Evidence for the genetic and physical recombination of the mitochondrial genomes of different species is also presented.

MATERIALS AND METHODS

The general culture methods used were based on those given in reference 31 and for the culture and crossing of the antibiotic resistant strains see references 5, 17, 18 and 21. The methods for lytic enzyme preparation and protoplast isolation and fusion were slight modifications (32) of those given in references 33, 34, 35 and 36. The methods of extraction and purification of mitDNA, its treatment with restriction endonucleases and agarose gel electrophoresis were based on those of Stępień et al (23). Detailed descriptions of the experiments will be published elsewhere. The strains were as follows:

INTRASPECIFIC TRANSFER AND RECOMBINATION OF MITOCHONDRIA

A. nidulans - mutant derivatives of Glasgow strains (NRRL 194):

7-28, pyroA5 7-29, pabaA6 7-48, biA1; AcrA1, wA3; nicC10

7-161, wA3; pyroA4; (camA112) 7-163, pyroA5 (oliA1)

7-172, pabaA1, yA2; blA4 (camA112) 7-178, pabaA6 (oliA1)

See reference 31 for the designation of the mutant alleles. All of these strains carry the hetA1 and hetB1 heterokaryon incompatibility alleles.

A. nidulans - progeny of inter-isolate cross, Glasgow x Birmingham isolate 1:

JC.12-8, pabaA6, yA2; hetA2; hetB1

JC.12-59, pabaA6, yA2; hetA1; hetB2

A. quadrilineatus - Mutant derivatives of Birmingham isolate 12:

12-15, y-12.1, pyro-12.1 12-22, y-12.1, pyro-12.1 (oli-12.7)

12-39, nic-12.1 (oli-12.7)

A. nidulans var. echinulatus - Mutant derivative of Birmingham isolate 25:

25-2, y-25.2

Interspecific mitochondrial transfers or recombinants:

RD.11-1, y-12.1, pyro-12.1 (oliA1) See Table 1(i)

JC.16-10, pabaA6 (oli-12.7) See Table 1(ii)

JC.17-5, y-25.2 (oliA1) See Table 1(iii)

JC.19-1, pabaA1, yA2; blA4 (oli-12.7, camA112) See results section.

RESULTS

Transfer of mitochondrial markers between heterokaryon incompatible strains of A. nidulans.

Preliminary protoplast fusion experiments were carried out between an oligomycin resistant strain of Glasgow origin, 7-163, and oligomycin sensitive strains which differed from the Glasgow strain either at the hetA gene, JC.12-8, or at the hetB gene, JC.12-59. (For a discussion of heterokaryon incompatibility see Dales and Croft, this volume). Dilutions of the fused protoplast suspensions were plated on minimal medium and heterokaryons typical for hetA and hetB heterozygosity respectively were recovered (24, 37). Conidial suspensions were obtained from these heterokaryons and plated on medium containing p-aminobenzoic acid and oligomycin and yellow spored, p-aminobenzoic acid requiring strains, now resistant to oligomycin were recovered.

Interspecific transfer of mitochondrial markers

Interspecific protoplast fusion experiments do not always produce stable heterokaryons as the primary selected fusion product. Therefore, in these experiments, selection for the required genotype resulting from the transfer of the cytoplasmic marker was carried out directly on the fused protoplasts. The experiments performed are listed in Table 1(a). Also listed in Table 1(b) are two experiments where (oliA1) has been transferred from A. nidulans to another species and then transferred back to

A. nidulans again. The frequency with which the transfer of the mitochondrial marker took place was variable and ranged from about 0.3% to about 5% in terms of the number of selected oligomycin resistant colonies which regenerated per total number of colonies with the 'recipient' nuclear genotype which regenerated in the absence of oligomycin.

Table 1 Transfer and re-transfer of mitochondrial markers between different species of Aspergillus

STRAINS	SELECTION MEDIUM	RESULTANT TRANSFER
(a) Transfers:		
(i) 7-178 + 12-15	pyridoxine and oligomycin	(oliA1) from nidulans to quadrilineatus
(ii) 12-22 + 7-29	p-aminobenzoic acid and oligomycin	(oli-12.7) from quadrilineatus to nidulans
(iii) 7-178 + 25-2	oligomycin	(oliA1) from nidulans to var. echinulatus
(b) Re-transfers:		
(iv) RD.11-1 + 7-29	p-aminobenzoic acid and oligomycin	(oliA1) from quadrilineatus to nidulans
(v) JC.17-5 + 7-48	biotin, nicotinic acid, acriflavine and oligomycin	(oliA1) from var. echinulatus to nidulans

Interspecific recombination of mitochondrial genes

In the first experiment a nicotinic acid requiring strain of A. quadrilineatus carrying the (oli-12.7) mitochondrial allele, 17-39, was fused with a yellow spored, p-aminobenzoic acid requiring strain of A. nidulans carrying the (camA112) mitochondrial allele, 7-172. Fused protoplasts were plated on selection media containing oligomycin, chloramphenicol and either nicotinic acid or p-aminobenzoic acid in order to select the recombinant mitochondrial phenotype in either nuclear background. One such strain, JC.19-1, was recovered and this has the nuclear genotype of 7-172. Strain JC.19-1 is fully heterokaryon compatible with the Glasgow strains and it was put into a heterokaryon with 7-28. Conidia from this heterokaryon were plated on media containing pyridoxine and either single antibiotic. Upon testing, all 32 green spored, pyridoxine requiring colonies isolated from the oligomycin medium and 31 of the 32 isolated from the chloramphenicol medium were found to be resistant to both antibiotics, strongly suggesting that both resistance genes are located on a single genetic element. The other colony isolated from the chloramphenicol medium was resistant to chloramphenicol only and it presumably represents a further recombination event or possibly a new spontaneous mutation arising in 7-28.

In the second experiment JC.16-10, an A. nidulans strain to which the A. quadrilineatus mitochondrial mutation (oli-12.7) has been transferred by

protoplast fusion, was put into a heterokaryon with 7-161. Conidia from this heterokaryon were plated onto medium containing pyridoxine, p-aminobenzoic acid, oligomycin and chloramphenicol. Many well conidiating colonies grew on this medium in addition to a background of non-conidiating colonies. A sample of 32 white spored and 24 green spored colonies was taken and tested. Of the 32 white spored colonies all had the 7-161 nuclear genotype and 26 were resistant to both antibiotics. The other 6 were chloramphenicol resistant only and therefore represent the 7-161 parental type. Of the 24 green spored colonies all had the JC.16-10 nuclear genotype and 17 were resistant to both antibiotics. The remaining 7 were resistant to chloramphenicol only and represent a mitochondrial transfer type. The background of non-sporulating colonies proved to be resistant to oligomycin only and to represent the JC.16-10 parental type and the 7-161 mitochondrial transfer type in about equal proportions. Fully selective procedures were used here to obtain the doubly resistant recombinant class and this occurred at a frequency of about 10^{-5} among the total conidia plated. In Aspergillus the frequency of recombination of mitochondrial genes has been expressed in terms of the ratio of the number of recombinants to the number of individuals showing reassortment between nuclear and cytoplasmic markers (5). In the present experiment, if it is assumed that half of the non-conidiating background colonies represent one of the two reassorted types and that these two types actually occur in equal proportions, then the doubly resistant recombinants occurred with a frequency of about 2.5% among all conidial progeny showing reassorted phenotypes. This is low compared with the results of Rowlands and Turner for recombination between (oliA1) and (camA112) in A. nidulans (21) and should not be taken as indicating close linkage of (oli-12.7) and (camA112). Experiments specifically designed to estimate the frequency of recombination between (oli-12.7) and (camA112) and to test for the allelism of (oliA1) and (oli-12.7) have not yet been performed.

Physical recombination of the mitochondrial genome

The analysis of the restriction endonuclease fragments produced by the digestion of mitDNA from strains of A. nidulans, A. quadrilineatus and A. nidulans var. echinulatus with a range of enzymes, particularly HindIII, HaeIII, HhaI and EcoRI, shows there to be a considerable degree or relationship between the three species but also reveals a number of differences between them. The total molecular weights for the mitDNA of the three species, as estimated by the use of standard DNA of known molecular weight, are about 20.5, 19.5 and 23.5 million daltons respectively. Though the analysis of the mitDNA of the genetic recombinants has not been carried out, a number of strains resulting from the interspecific transfer of mitochondrial genes have been examined. These are RD.11-1 (A. nidulans (oliA1) transferred to A. quadrilineatus nuclear background), JC.16-10 (A. quadrilineatus (oli-12.7) transferred to A. nidulans nuclear background) and JC.17-5 (A. nidulans (oliA1) transferred to var. echinulatus nuclear background). In the case of RD.11-1 the mitDNA was indistinguishable from that of A. nidulans and the transfer of the whole mitochondrial genome is a sufficient explanation for the origin of this strain. However, the mitDNA both of JC.16-10 and of JC.17-5 show non-parental fragment patterns. A second strain of each of these classes of transfer has recently been examined and, again, both show non-parental, but different, patterns. The mitDNA of JC.17-5 has been analysed in the greatest detail so far (Fig 1). It has a molecular weight of about 21.8 million daltons, intermediate between that of both

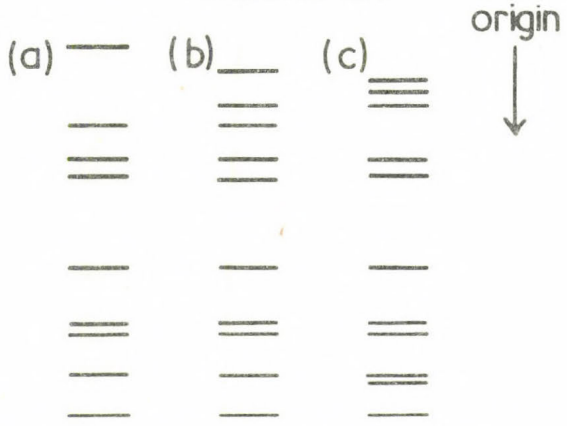

Fig.1. Banding patterns produced by the electrophoresis of HindIII digests of mitDNA of (a) A. nidulans, (c) A. nidulans var. echinulatus, and (b) a hybrid of these two species.

parents, and its origin can tentatively, and most simply, be explained by recombinational events at two regions resulting in a genome derived about equally from both parents.

COMMENTS

These experiments demonstrate that the technique of protoplast fusion facilitates the transfer of mitochondrial genomes between taxonomically related, but quite distinct, species of Aspergillus. The apparent lack of variation in the physical structure of the mitochondrial genome of A. nidulans, together with the low number of mutant mitochondrial loci, make the relative ease with which the structurally different mitochondrial genomes of the three species can be brought together to interact genetically of some importance for the study of organelle genetics in this genus. Recombinant mitochondrial genomes are produced without their direct selection merely by the selection for the transfer of a single marker from one species to the other, and this occurs apparently with a high frequency. It is of interest to note that this took place only in those cases where the smaller mitochondrial genome was being selected for transfer to the species which normally contains a larger genome. In the one case where the reverse was attempted it appeared that the whole mitochondrial genome was transferred unrecombined, but the significance of this requires further investigation. In the Paramecium aurelia group of species, where interspecific transfer of mitochondria has been achieved by microinjection, there was evidence of incompatibility between some combinations of mitochondrial genome and nuclear background (38). The authors suggested a number of explanations for this observation, one of which was the lack of mitochondrial recombination in those species. It may well be that the lack of such incompatibility in Aspergillus is due to the apparent ease with which mitochondrial recombination takes place.

SUMMARY

Protoplast fusion has been used to transfer antibiotic resistant mitochondrial markers between heterokaryon incompatible strains of A. nidulans and between different species of Aspergillus and also to bring

about genetic recombination between pairs of antibiotic resistant mitochondrial markers derived from different species. The physical recombination of mitDNA from different species has been demonstrated by the analysis of restriction endonuclease fragments.

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PROTOPLAST FUSION IN THE STUDY OF MITOCHONDRIAL GENETICS IN THE PETITE-NEGATIVE YEAST, KLUYVEROMYCES LACTIS

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Summary

Sporulation-deficient diploids of K.lactis can be isolated by protoplast fusion between heterothallic haploid strains of like mating-type. The isolation of stable diploids by protoplast fusion provides a means of overcoming the problem of unstable diploidy in this yeast species. The possibility of using protoplast fusion to study mitochondrial inheritance in K.lactis has been investigated.

Introduction

In heterothallic strains of the petite-negative yeast, Kluyveromyces lactis, mating takes place only between cells of opposite mating-types (1) producing diploids that are heterozygous (a/α) for mating-type. These diploids are, however, unstable and will eventually sporulate on any medium. Unstable diploidy is most evident in mating-products derived from crosses between petites and wild-type cells of K.lactis such that, in certain strain combinations, only haploid recombinants are recovered (2).

By protoplast fusion between heterothallic strains of like mating-type, it has been possible to isolate stable hybrids that are sporulation-deficient (3). The protoplast fusion technique has been applied to both wild-type (ρ^+) and petite (ρ^-) cells. Compared to haploid parentals, hybrid cells are uniformly of diploid size and carry approximately twice the DNA content. Nuclear staining reveals the presence of only one nucleus per hybrid cell (4). In addition, a small proportion of haploid recombinants are isolated.

Sporulation-competent hybrids have been constructed by protoplast fusion between homothallic strains of K.lactis (4). These "homothallic hybrids" can be distinguished from sexually produced diploids by their very low levels of sporulation (<2%). Tetrad analysis shows a 2:2 segregation of nuclear markers.

The evidence suggests that hybrids isolated by protoplast fusion are predominantly diploid. The possibility of using protoplast fusion as an efficient method for isolating sporulation-deficient hybrids of K.lactis could provide a means of overcoming the problem of unstable diploidy in this yeast species. This could prove to be particularly useful in the

study of extra-chromosomal inheritance in K.lactis.

In this paper, we investigate the possibility of using protoplast fusion to study mitochondrial genetics in K.lactis.

Materials and Methods

1. Strains of K.lactis

Strain	Phenotype
102	a arg
106	a arg ery^R
110	a trp
127	a argtrp ery^R

These strains are heterothallic and the deficiences in arginine biosynthesis of strains 102 and 127 are complementing. By tetrad analysis, it has been shown that in 106, ery^R is determined by an extrachromosomal mutation and in 127, ery^R is conferred by a single dominant nuclear gene(5).

2. Media

Media composition is given in Morgan and Whittaker (3,6).
MMA = minimal medium agar. OMMA = as for MMA except 0.6M KCl is added for osmotic stabilisation of protoplasts. MMY = as for MMA except glucose is replaced by 2% (v/v) glycerol. MMYE = MMY + 4 mg/ml erythromycin.

3. Petite induction

The petite mutation was induced with ethidium bromide (EB) according to the method of Heritage and Whittaker (7).

4. Protoplast fusion.

Hybrids ($ery^R + ery^S$)were isolated by the protoplast fusion procedure described by Morgan and Whittaker (3). For each of the ery^R mutants, hybrids were isolated from the following crosses:-

(i) $\rho^+ ery^R + \rho^- ery^S$
(ii) $\rho^- ery^R + \rho^+ ery^S$
(iii) $\rho^+ ery^R + \rho^- ery^S$

5. Mitotic Segregation Analysis

The procedures used for mitotic segregation analysis were based on the technique of zygote clone analysis (8). A hybrid colony issued from a single fusion product is defined as a primary clone. The four principal steps of the analysis are shown diagrammatically in Fig.1. For the qualitative analysis, more than 20 primary clones were isolated into 1 ml sterile water and patched onto MMA to eliminate parentals or recombinant auxotrophs (step 1). After 5 days incubation at 30°C, the patches were replica-plated onto MMY, MMYE, and MMA (step 2). After a further 4 to 5 days incubation, growth was scored. For the quantitative analysis, the MMA replicas were suspended in sterile water, counted, diluted and spread onto MMA to allow growth of secondary clones (step 3). After 4 days incubation at 30°C, the secondary clones (up to 60 per hybrid) were replica-

plated with sterile toothpicks onto MMY and MMYE (step 4). After 3 days incubation, clonal growth was scored as positive or negative. A positive score was given even when only a single papilla was formed on MMYE.

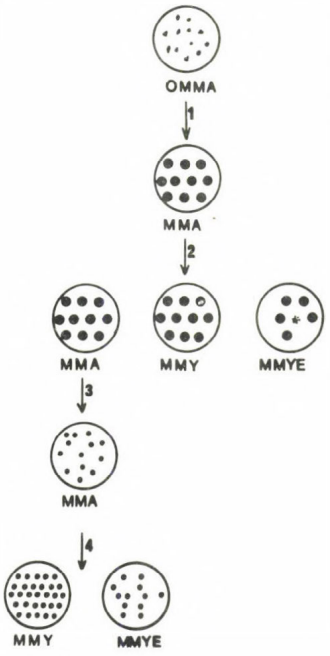


Fig.1. Diagrammatic representation of mitotic segregation analysis procedures.Hybrids were selected in OMMA,isolated and assessed qualitatively(steps 1 and 2)and quantitatively(steps 3 and 4)for ery^R/ery^S

Results and Discussion

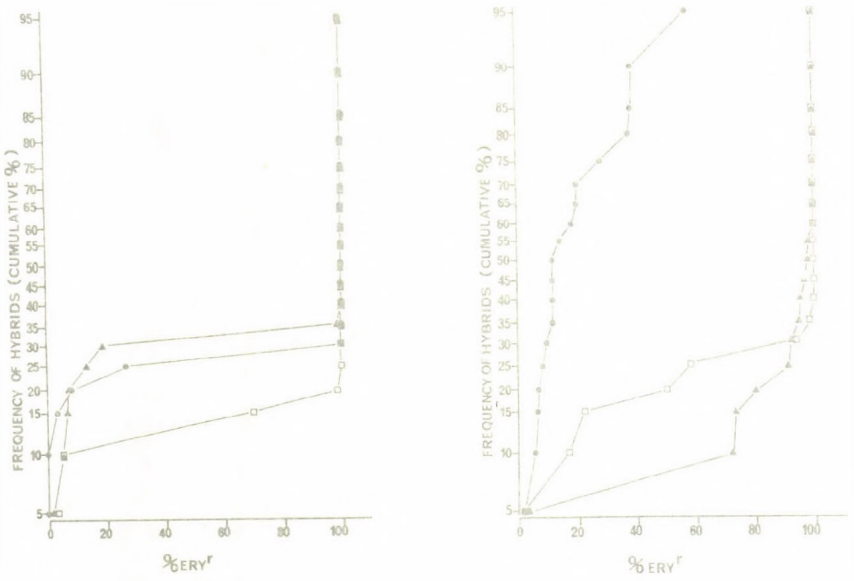
In Saccharomyces cerevisiae, mitochondrial genes have been characterised by three criteria :(i) non-Mendelian inheritance, (ii) mitotic segregation, and (iii) deletion by treatment with petite mutagens. In the case of K.lactis, however, the unstable diploid phase permits only the first of these criteria to be used when conventional techniques of analysis are employed. Nevertheless the construction of stable hybrids by protoplast fusion between strains of like mating-type offers the possibility of undertaking mitotic segregation and petite deletion analysis in K.lactis. Qualitative and quantitative procedures of analysis have been used.

Qualitative analysis of the inheritance of ery^R as a nuclear gene (strain 127) shows that a small proportion of primary clones exhibit sensitivity to erythromycin.

TABLE 1. Qualitative Analysis of Hybrids, ery^R + ery^S

Protoplast Fusion Cross	No of Primary Clones ery^R score				Total No. Primary Clones Tested
	-	+	++	+++	
106 + 110	0	2	2	17	21
106 ρ^- + 110	6	14	0	0	20
106 + 110 ρ^-	0	0	3	18	21
127 + 102	0	4	2	18	24
127 ρ^- + 102	1	6	1	17	25
127 + 102 ρ^-	2	2	2	17	23

These sensitive primary clones were presumed to be haploid recombinants. This assumption was confirmed by qualitative assessment of cell size (4). The quantitative analysis (Fig.2a) shows that the hybrids fall into two distinct classes (ery^R and ery^S). Since only a small proportion of haploid recombinants are isolated in a protoplast fusion cross(4), the majority class (ery^R) will consist mainly of diploid hybrids. Treatment of one or other of the parental types (127 or 102) with EB has little or no effect on the distribution of resistance or sensitivity amongst primary and secondary clones. These results are consistent with those obtained by tetrad analysis(5). That is, ery^R in 127 is conferred by a dominant nuclear gene.



(a) (b)

Fig.2. Quantitative analysis of hybrids derived from fusion crosses involving strain 127 (Fig.a) and strain 106 (Fig.b). ρ^+ery^R + ρ^+ery^S (□); ρ^-ery^R + ρ^+ery^S (●); ρ^+ery^R + ρ^-ery^S (▲)

In strain 106, resistance is determined by an extrachromosomal gene, which is probably located on the mitochondrial genome (see below). Qualitative analysis shows that a small proportion of primary clones ($\rho^+ + \rho^+$) are sensitive (Table 1). These may represent haploid recombinants or diploids in which mitotic segregation of homoplasmic progeny (ery^S) occurred at an early stage following the formation of the initial protoplast fusion product. Secondary clone analysis reveals that in a high proportion (63.2%) of the hybrids ($\rho^+ + \rho^+$) only resistant progeny were obtained (Fig. 2b). Since the mechanisms of mitochondrial inheritance in K.lactis are not well understood, it is not possible to tell whether this observation suggests a strong bias in the transmission of ery^R alleles or a long-term maintenance of the heteroplasmic condition. Treatment of 106 with EB prior to fusion with 110 produces a clear shift in favour of sensitive progeny as observed by qualitative and quantitative procedures of analysis. Since EB acts specifically on mitochondrial DNA, the effects on transmission would suggest that the resistance allele is located on the mitochondrial genome. That EB has a direct effect on mitochondrial gene transmission is demonstrated by the reciprocal cross in which there is a shift towards resistant progeny. It is not possible to tell whether a reduced transmission is a consequence of a lowered mitochondrial gene content in petites resulting in a decreased input, or the result of mitDNA fragmentation by EB and subsequent effects on recombination processes. The results do suggest however, that in K.lactis there are multiple copies of genetically competent and identical mitochondrial genomes.

In conclusion, the isolation of stable (sporulation-deficient) diploids by the technique of protoplast fusion provides a means of undertaking mitotic segregation and petite deletion analysis in K.lactis. For more advanced studies on the transmission, segregation, and recombination of mitochondrial genes in K.lactis it will be necessary to eliminate the haploid recombinant products of protoplast fusion. In fact, haploids and diploids are readily distinguished by their colony colours on minimal media (3).

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GENETIC STRUCTURE OF YEAST HYBRIDS CONSTRUCTED BY PROTOPLAST FUSION

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Bypassing natural barriers of sexual hybridizations, protoplast fusion can be a powerful tool in both pure and applied yeast genetics. To realize this opportunity, detailed knowledge about the genetic consequences of protoplast fusion in yeasts is essential.

The first successful hybridizations of yeasts by protoplast fusion were reported in 1977 (1-6). In these and some following investigations it was demonstrated that protoplasts from auxotrophic strains of a yeast species will hybridize despite identity in mating type. Since the isolated prototrophic fusion products were genetically stable and heterozygous for the parental markers, the DNA content per cell was nearly twice as big as that of the parental strains, and only one nucleus per cell was found, they seem to be similar if not identical to sexual hybrids. Such results were obtained in taxonomically different yeasts, e.g. with *Saccharomyces cerevisiae* (1,2,7), *Schizosaccharomyces pombe* (3) and *Rhodosporidium toruloides* (4). However, in experiments with *Candida tropicalis*, the isolated fusion products were mainly genetically unstable heterokaryons which give rise to stable hybrids only during further cell propagation cycles (6,8).

Using yeast strains with suitable chromosomal markers, protoplasts of log phase cells prepared by *Helix pomatia* enzymes, and the polyethylene glycol fusion technique, we have studied genetical consequences of protoplast fusions in *Pichia guilliermondii*, *Saccharomyces cerevisiae* and *Rhodosporidium toruloides* (9-11). The fusion products (which we call fp-hybrids) were mainly studied by mitotic segregation, and in the case of *S. cerevisiae*, tetrad analysis was also used. To increase mitotic segregation frequencies, usually the chromosome loss inducing agents acriflavin and benomyl were used. The ability of acriflavin to increase the frequencies of mitotic segregation by chromosome loss was found in our laboratory. The following procedures of drug application and isolation of auxotrophic segregants were mostly used. After growth on complete medium agar with suitable drug concentrations, the colonies, each from a single cell, were replica plated onto minimal medium agar. In this case, each segregant colony results from one segregation event.

In this paper, some results are presented which demonstrate that the genetical consequences of protoplast fusions in yeasts may be more complicated than expected from previous investigations.

High Mitotic Segregation Frequency of One of the Parental Markers in fp-Hybrids

The majority of the fp-hybrids within intraspecific protoplast fusions in *P. guilliermondii*, *S. cerevisiae* and *R. toruloides* are perhaps heterozygous for all parental markers, since we recovered the markers from those hybrids tested (examples are presented in the tables). The heterozygous state of most fp-hybrids is stably transmitted during vegetative propagation. The spontaneous mitotic segregation frequency of the markers varied considerably, but in most cases a frequency of 10^{-3} or less was obtained.

In some fp-hybrids, however, a much higher mitotic segregation frequency was found for one of the parental markers, whereas, the others are in a stable heterozygous state. This surprising phenomenon was observed in fp-hybrids from protoplast fusions between mutants of different wild strains of *R. toruloides* and *P. guilliermondii*. It is characterized by the following three examples.

Ten prototrophic fp-hybrids were isolated within a protoplast fusion experiment between the *Rhodosporidium* strains IFO 559 A leu and CCY 20-2-16 A met. Eight of these hybrids were stable prototrophs: auxotrophic segregants were not found among 1000 tested colonies of each hybrid. The other two hybrids segregate met with a high spontaneous frequency: 530 met segregants were found among 1200 tested colonies of one of the hybrids and 100 met segregants among 500 tested colonies of the other hybrid.

Similar results were observed with prototrophic fp-hybrids within protoplast fusions between the *Rhodosporidium* strains IFO 559 A leu pan and CCY 20-2-16 A met ade. Two of the hybrids segregate ade with a high frequency: 17 % and 90 % of the tested colonies were ade segregants in one of the experiments. With a much lower frequency the marker combination ade met also segregates spontaneously. The high spontaneous segregation frequency of ade was not eliminated during the vegetative propagation of the unstable fp hybrids.

Seven out of eight tested prototrophic hybrids within a protoplast fusion experiment between the *P. guilliermondii* strains 799 mat⁻ met nic and 809 mat⁻ his ade were stable heterozygots: Among approximately 1000 tested colonies of each fp-hybrid, auxotrophic segregants were not observed. One of the hybrids segregates spontaneously the marker ade only, which can be easily observed by the red colour of ade colonies or colony sectors. Out of 909 tested colonies of the fp-hybrid, 19 were completely red while most others had red sectors (197 red sectors per 300 tested colonies). The high spontaneous mitotic segregation frequency of the marker ade was eliminated during some vegetative reproduction cycles of the cells.

These results suggest that in rare protoplast fusion events, single chromosomes or pieces of a chromosome were not stably integrated into the hybrid nucleus, but exist as a plasmid-like genetic element in the cell (nucleus?). This may be a transient or permanent condition . Perhaps, the integration probability into the nuclear genomes is a characteristic feature of such plasmid-like elements.

Preferential Mitotic Co-segregation of Unlinked Parental Marker Combination in Stable Hybrids of Protoplast Fusions Between the Strains 799 met arg and 799 asn his of *P. guilliermondii*

Unlinked markers segregate independently in most sexual hybrids, but not in many fp-hybrids. The hybrids produced by protoplast fusions between the mutants met-1 arg-3 and asn-1 his-7 of the *P. guilliermondii* wild strain 799 (mating type mat$^-$) showed preferential co-segregation of met with arg and of asn with his, although these markers are unlinked and the hybrid cells have only one nucleus. Linkage could be excluded by independent mitotic segregation of the auxotrophic markers in hybrids produced by sexual crosses and in other fusion combinations. The data of UV light induced mitotic segregation presented in Table 1 demonstrate also that the four markers are unlinked. The genetic stability of the fp-hybrids and the occurrence of some recombinants with genetic markers from both parents exclude a heterokaryotic condition. Evidence for the monokaryotic state was also found in light and electron microscopic studies. Data of three fp-hybrids are presented in the following table.

Table 1 Spontaneous and induced mitotic segregation data

Fusion hybrid	Frequency of spontaneous mitotic segregants	Induced mitotic segregation				
		Inducing agent	No. of segregants tested	No. of segregant types		
				met arg	asn his	others
fp3-33	3×10^{-3}	acriflavine	31	11	19	1 asn met
fp3-51	1×10^{-3}	acriflavine	48	28	20	0
fp3-70	5×10^{-3}	acriflavine	51	15	35	1 met
		benomyl	163	92	67	4 met
		UV light	34	3	2	8 asn
						7 his
						7 met
						7 arg

The co-segregation which was induced also by p-fluorophenylalanine occurs only in the fusion combination 799 met arg + 799 asn his, but not in combinations of one of these mutants with other mutants of strain 799. The following DNA amounts per cell were determined: 26 + 3 and 28 + 6 fg in the two parental mutants, 44 ± 2 fg in the fp-hybrid 3-70, 31 ± 1 and 27 ± 4 fg in two co-segregants of fp3-70.

These experimental data suggest that karyogamy occurs after protoplast fusions between the mutants 799 met-1 arg-3 and 799 asn-1 his-7. The unusual, but comparatively stable organization of the resulting synkaryon allows recombinations between the two genomes, which have, however, retained a certain degree of independence.

The Mitotic Segregation Pattern of Stable fp-Hybrids May Be Different Within a Protoplast Fusion Experiment

An example is given in Table 2. In the three presented prototrophic hybrids of protoplast fusions between the *P. guilliermondii* strains 799 mat⁻ met nic and 809 mat⁻ his ade, the spontaneous

Table 2 Acriflavine induced mitotic segregation patterns of *P. guilliermondii* fp-hybrids

Types of segregants	Frequency in % of the total number of segregants		
	fpI-10	fpI-II	fpI-61
met	27.6	23.1	4.2
nic	0	0.3	0
his	30.9	40.0	64.9
ade	4.6	7.2	3.2
met nic	5.3	0.3	3.2
his ade	2.6	5.0	11.7
met his	18.4	21.2	2.1
met ade	2.0	0	2.1
nic his	0.5	0	1.0
nic ade	1.2	0	0
met nic his	0.6	0.3	0
met nic ade	0.6	0	1.0
his ade met	5.3	3.0	5.3
his ade nic	0	0	1.0
met nic his ade	0	0	0
Number of segregants tested	152	363	94

segregation frequencies of auxotrophic markers is lower than 10^{-3}, but mitotic segregants could be obtained by means of the chromosome loss inducing agent acriflavine.

Whereas, the segregation patterns are similar in the hybrids fp1-10 and fp1-11, significant differences exist between these two fp-hybrids and fp1-61 in the frequencies of met, his, his ade, and his met segregants. These results indicate differences in the genetic structure of the respective fp-hybrids.

Tetrad Analysis of fp-Hybrids of *S. cerevisiae*

To get detailed information about the genetic organization of stable fp-hybrids, protoplast fusions and sexual crosses between the *S. cerevisiae* strains H10/6 a ade5 aro2 cyh2 leu1 ade2 and H10/10 α his4 lys5 met13 trp5 ade2 were performed.

The mating type alleles and his4 are located in chromosome III, ade2 in chromosome XV, and the other loci in chromosome VII. After sporulation, the tetrads of sexual and fp-hybrids were analysed by means of the micromanipulator.
All five tested fp-hybrids are heterozygous diploids with a normal 2:2 segregation of the markers in most cases. However, some differences to the sexual hybrids and perhaps also between fp--hybrids exist (Table 3).

Table 3 Tetrad data of the sexual hybrids (70 tetrads) and the fusion product 15 (34 tetrads)

Marker	Number of tetrads with wild:mutant allele ratio					% of aberrant tetrads
	4:0	3:1	2:2	1:3	0:4	
			Sexual hybrids			
his4	0	1	68	0	1	2.8
ade5	0	1	38	0	0	2.5
lys5	0	2	68	0	0	2.8
aro2	0	0	69	1	0	1.4
met13	0	2	68	0	0	2.8
cyh2	0	0	69	1	0	1.4
trp5	0	0	70	0	0	0
leu1	0	0	68	2	0	2.8
			fp 15			
his4	1	4	29	0	0	14.7
ade5	0	3	31	0	0	9.7
lys5	1	3	30	0	0	13.3
aro2	0	6	28	0	0	17.6
met13	1	4	29	0	0	14.7
cyh2	0	0	31	3	0	9.7
trp5	0	5	29	0	0	14.7
leu1	0	4	30	0	0	13.3

The frequency of aberrant tetrads is significantly higher in the fp-hybrid than in the sexual hybrids. While in the sexual hybrids the frequencies of conversion from the mutant to the wild and from the wild to the mutant alleles tends to be equal at given loci, these frequencies are significantly unequal in fp 15, with a strong bias in the direction of the wild type allele.

SUMMARY

In intraspecific protoplast fusion experiments between auxotrophic strains of *Pichia guilliermondii*, *Saccharomyces cerevisiae* and *Rhodosporidium toruloides*, the majority of isolated prototrophic products (fp-hybrids) are genetically stable. However, we found also fp-hybrids with a high spontaneous mitotic segregation frequency of one or more parental makers. Although a heterokaryotic condition could be excluded, the protoplast fusions between two strains give rise only to fp-hybrids with preferential mitotic co-segregation of unlinked parental marker combinations by chromosome loss inducing agents. Many mitotically stable fp-hybrids are different from sexual hybrids and also from another within the same combination of strains. Obviously, the genetic consequences of protoplast fusions in yeasts may be more complicated than those of sexual cell unions.

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HYBRIDIZATION OF *RHODOSPORIDIUM TORULOIDES* BY PROTOPLAST FUSION

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INTRODUCTION

The genus *Rhodosporidium*, established by Banno in 1967 has considerable advantages for genetic studies over other basidiomycetes because of its ability to grow as a yeast on simple media and to complete the life cycle under these conditions. Therefore such studies were performed in our laboratory on *R. toruloides*. It was possible to develop effective techniques for induction and isolation of mutants, complementation and dominance tests in heterokaryons and heterozygotes, sexual hybridization and the analysis of sexual and mitotic segregation. Many strains with suitable chromosomal markers were constructed and are available.

Sexual crosses are possible only between yeast strains of opposite mating types. After the conjugation of two cells a dikaryotic mycelium with clamp connections and teliospores is formed. The teliospores are the site of karyogamy and germinate to a promycelium on which monokaryotic yeast cells, the sporidia, bud off. The formation of the sporidia on the promycelium is connected with the segregation of genetic markers, but the segregation patterns are abnormal and perhaps nonmeiotic (2).

Since the natural barrier of sexual conjugation can be eliminated by protoplast fusion; this method may be a powerful tool in *Rhodosporidium* genetics. Sipiczki and Ferenczy have first shown that it is possible to hybridize strains of the mating type A via protoplast fusion (3). We have studied the genetic consequences of protoplast fusions between yeast strains both of identical and opposite mating type. Some results of these experiments are presented in this paper.

MATERIALS AND METHODS

Double auxotrophic mutants of the wild strains Rgl and IFO 880 with mating type a and of IFO 559 and CCY 20-2-16 with mating type A were used. The reversion frequency of each of the auxotrophic markers was generally less than one per 10^8 cells. Pro-

toplasts have been prepared with the help of snail digestive juice as described by Hedenström and Höfer (4). Early log-phase cells were used and it was necessary to vary the cultivation time in liquid medium because auxotrophic mutants differ in their growth characteristics. Protoplast suspensions have been washed twice with 1 M sorbitol, mixed and centrifuged at 3000 x g. The pellet was suspended in a solution of 30 % (w/v) PEG in 0.1 M $CaCl_2$ and 15 % dimethyl sulphoxide. After 30 minutes incubation at 28°C the suspension was spread on fusion agar (minimal medium agar (5) stabilized osmotically with 0.8 M mannitol). Prototrophic colonies developing on fusion agar were regarded as fusion products. Fusion yield is compared with the number of protoplast pairs which are able to regenerate on supplemented fusion agar. DNA content of the strains was determined by the method of Burton (6) modified after (7). In this case cells have been washed twice with 10 % TCA and ethanol before $HClO_4$ extraction. For nuclear staining the Giemsa-method was used. Mitotic segregation, induced by acriflavine (8), and detection of mating type (9) were performed as described.

RESULTS

The yields of fusion products (fp-hybrids) were low and varied in different fusion experiments.

Fusions Between Different Mutants of the Same Strain with Mating Type a

In the fusions between different mutants of the same strain with mating type a very stable hybrids were formed (Table 1 and 2).

Table 1. Some characteristics of fp-hybrids of fusion No.3: IFO 880 a arg leu+IFO 880 a lys met (fusion yield: 1×10^{-4} %), DNA content of the strain IFO 880 a:67 fg per cell

1 fp-Hybrid	2 Mating type	3 DNA content in fg/cell	4 No. of spontaneous segregants/No.of colonies tested	5 Acriflavine induced segregants/ /No.of colonies tested
fp2-2	a	211	0 : 1000	24 met 1 arg 5 leu : 1000 2 lys 1 arg leu lys met
fp2-3	a	203	0 : 1000	7 met 1 arg 1 leu : 1000 1 lys

We succeeded in the induction of mitotic segregants by acriflavine. The results in the tables demonstrate that the prototrophic fp-hybrids were heterozygotic for the parental chromosomal markers.

Table 2. Some characteristics of fp-hybrids of fusion No.5: Rg 1 a ura leu + Rg l a lys ade (fusion yield: $1x10^{-3}$ %) DNA content of the strain Rg 1 a : 65 fg per cell

1	2	3	4	5
fp5-3	a	130	4 leu : 1000	14 leu 36 ura 2 ade : 1000 1 ade lys 1 ura leu
fp5-4	a	134	0 : 1000	2 leu 1 lys 1 ura leu : 1000 1 ade leu

In more than 200 fp-hybrids investigated no change of mating type from a to A or mycelium formation could be detected. In fusion No. 3 fp-hybrids seem to have a three times higher content of DNA compared with the parental strain whereas fp-hybrids of fusion No. 5 only contain double that of their parental strain. Nuclear staining showed that a few yeast cells with two nuclei occur, whereas most cells contain only one nucleus. Perhaps the balance between nuclear and cell division is disturbed in some yeast cells. For mycelium formation it seems likely that cells should carry opposite mating type alleles.

Fusion Between Mutants of Different Strains with Identical Mating Type

Hybrids of this fusion type were quite different, especially in genetic stability and DNA content. For instance fp2-4 is a very unstable fp-hybrid, whereas fp2-2 is a very stable one. But in no case a change or loss of mating behaviour in the hybrids or their auxotrophic segregants could be detected.

Hybrids of fusion between two strains with mating type A do not grow as mycelium.

Table 3. Some characteristics of fp-hybrids of fusion No. 2: IFO 559 A leu pan + CCY 20-2-16 A met ade (fusion yield: 2×10^{-3} %), DNA content of IFO 559: 97fg per cell and CCY 20-2-16: 95 fg per cell

1	2	3	4	5
fp2-1	A	252	0 : 1000	2 ade 1 leu : 1000
fp2-2	A	125	0 : 1000	2 ade 4 pan : 2000
fp2-4	A	94	110 ade: 1000	40 ade met. 1600 ade : 2000

Fusions Between Mutants of Different Strains with Opposite Mating Type

In fusion No. 6 and No. 7 (Table 4 and 5) four days after spreading the mixed protoplasts on fusion agar three types of prototrophic colonies developed: mycelium colonies, yeast colonies and mixed colonies which consist of mycelium and yeasts. Further investigation of the yeast types showed that hybrids with different mating types (a and A) have been formed. Cloning by micromanipulator showed that the mating type originally expressed is firmly inherited during vegetative reproduction. The viability (property of single hybrid cells to form a colony) is different.

Table 4. Some characteristics of fp-hybrids of fusion No. 6: IFO 880 a arg lys + IFO 559 A leu pan (fusion yield: 1×10^{-3} %)

1	2	3	4	5
fp6-1	A	150	2 pan : 2000	0 :2000
fp6-6	A	190	1 leu : 2000	4 leu :2000
fp6-7	a	129	2 lys 2 arg 3 arg lys : 2000	14 lys 1000 arg lys leu pan cys :2000

Table 5. Some characteristics of fp-hybrids of fusion No 7: IFO 880 a arg lys + CCY 20-2-16 A met ade (fusion yield: 6×10^{-4} %)

1	2	3	4		5		
fp7-2	A	190	2 met	: 2000	0	:	2000
fp7-4	Myz^+	365	0	: 2000	0	:	2000

The fp-hybrids differ in DNA content and the amount is not a simple addition of that of the parental strains. The fp-hybrids were genetically stable but in one case it was possible to induce a very high proportion of mitotic segregants which contained all four auxotrophic markers and additionally the cys-marker which was probably heterozygotic and situated in one of the parental strains. Observations of formation of colonies of fp-hybrids on fusion agar showed that the initial fusion product is yeast-like. After propagating by budding for a few times differentiation may occur. The complete turnover to dikaryotic mycelium could be detected. In addition, we found fp-hybrids developing as stable yeast colonies and some fp-hybrids remaining as potential mycelium forming yeasts (Myz^+) which produce mixed colonies of yeasts and mycelium. Nuclear staining by means of Giemsa-dye showed that the mycelium formed by Myz^+ strains is uninucleate. The Myz^+ property is genetically stable. On the uninucleate mycelium viable teliospores are formed which are able to germinate and to form sporidia. The Myz^+ yeast population is uninucleate, too .

DISCUSSION

The results show that in most cases stable fp-hybrids could be constructed by protoplast fusion. Genetic stability seems to be possible in hybrids possessing a wide range of DNA content. The data of mating behaviour, DNA content, genetic stability and the spectra of induced mitotic segregants show that quite different hybrids may arise from one fusion experiment. Probably fusions between more than two protoplasts may occur. It is assumed that in most cases immediately after fusion of strains with identical mating type a hybrid nucleus is formed which has to be balanced during the further divisions. It seems that the occurrence of genetically unstable fusion products is dependent on the fused strains. The results of fusions between strains of opposite mating type were surprising, because in sexual crosses, only dikaryotic mycelia are formed. The occurrence of stable uninucleate yeast-like fp-hybrids and Myz^+ hybrids leads to the assumption that for mycelium formation not only two different nuclei are

necessary but two nuclei in a special condition. The phenomenon that sporidia of *Rhodosporidium* species are able to form mycelium without conjugation is known from literature (1, 10). Since only in fusions between strains of opposite mating type Myz^+ hybrids are formed, it is assumed that the fp-hybrids are heterozygous for the mating type. Perhaps many fp-hybrids are stable aneuploids. Protoplast fusion and sexual cross of *Rhodosporidium toruloides* may lead to different products. As with sexual crosses protoplast fusion between strains of opposite mating types gives rise to dikaryotic mycelia but, in the latter case, only at a low frequency.

SUMMARY

PEG-mediated fusions between protoplasts of different mutants of the same strain, of different strains with identical mating type and of different strains with opposite mating type were analysed. The genetic consequences of protoplast fusions were studied by means of spontaneous and acriflavine-induced segregation, determination of mating types, and the DNA content per cell. It was found that mostly stable fp-hybrids can be formed but different hybrids can arise from one fusion experiment. Whereas in fusions between strains of identical mating type generally monokaryotic yeast cells occur, in fusions between strains of opposite mating type dikaryotic mycelium occurs too. In contrast to sexual crosses, however, yeast-like fp-hybrids were formed as well. The latter are in most cases uninucleate but also dikaryotic yeasts were found. Some of the fp-hybrids are yeasts which are able to form uninucleate mycelia.

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HYBRIDIZATION OF *PICHIA GUILLIERMONDII* BY PROTOPLAST FUSION

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Hybridization by protoplast fusion (1, 2) should be a useful method to enhance the ploidy level of yeasts in a parasexual way. We have performed a fusion programme between different yeast mutants and strains of *Pichia guilliermondii* with the intention to enhance the ploidy level by two or three times respectively.

In order to achieve this, we fused diauxotrophic mutants which possessed one similar mutation and got auxotrophic fusion hybrids. The latter were then fused with diauxotrophic mutants in order to produce prototrophic fusion hybrids with the threefold ploidy level of the parental strains.

The fusion hybrids were analysed by induced mitotic segregation. Their mitotic stability and their DNA content per cell were investigated.

Fairly stable prototrophic hybrids arose in the different fusion combinations. In the stable hybrid cells we did not find the expected high DNA content in each case. Therefore, the enhancement of the ploidy level by protoplast fusion appears to have limits in *Pichia guilliermondii*.

MATERIALS AND METHODS

Fusions were made with mutants of the strains *P. guilliermondii* 799 and 809, which we were kindly given by Dr Zsolt (Szeged) as the imperfect *Candida guilliermondii*. In our laboratory N.Prahl and I.A. Samsonova have found in mating experiments that these strains belong to *Pichia guilliermondii* and that they have the mating type mat^-.

Mutagenesis was performed by UV-light (3) and stable auxotrophic mutants with reversion frequencies lower than 10^{-7} were used for protoplast fusions.

Protoplasts were produced from log phase cells in protoplast medium (PPM), consisting of 50 mM Tris-HCl (pH 7.5) and 0.7 M $MgSO_4$, by 30 mg/ml lyophilized snail gut juice and 0.1 % 2-mercaptoethanol. After shaking between 2 and 3 hours at 30°C more than 99 % of the cells were converted to protoplasts. Intact

cells were removed by low speed centrifugation. For fusion, equal volumes of protoplast suspension of the two fusion partners were mixed and diluted with 50 mM Tris-CHl (pH 7.5) to decrease the $MgSO_4$ concentration to 0.25 M. It was then possible to separate protoplasts by centrifugation, and either PPM or 30 % polyethylene glycol (PEG) 4000 with 10 or 100 mM $CaCl_2$ was added to the pellet. After mixing, 0.1 ml of the protoplast suspension was spread on unsupplemented regeneration medium (solid yeast minimal medium containing 0.35 M $MgSO_4$ and 2 mg/ml biotin). The PPM-sample served as control for marker stability and spontaneous fusions, and the prototrophic fusion hybrids could be picked up from the PEG-plates after several days. One part of the PPM-sample was diluted in PPM and spread on regeneration medium containing supplements in order to control protoplast regeneration. Fusion products were cloned either by several passages on minimal medium or by micromanipulation.

Mitotic segregation was induced by 200 to 750 mg/ml acriflavine or by 150 mg/l benomyl (4).

DNA was measured in stationary phase cells (5).

RESULTS

Fusions were performed between diauxotrophic mutants of the strains 799 met-1 nic-2 and 809 his-1 ade-3. Moreover, a fusion programme was accomplished between different mutants of the strain 799.

Numbers of protoplasts and fusion yields of some experiments are shown in Table 1. The fusion yield is related to the number of protoplast pairs per ml which were able to regenerate and to form colonies.

The cell densities of each fusion partner used for one experiment was between 3.0×10^8 and 1.7×10^9 per ml. In some cases regeneration was low and varied from one experiment to another. Perhaps because of this variation there is no evidence for relations between certain fusion combinations and their fusion yield.

In the case of some fusion combinations, we were not able to get fusion products (data not shown). It is not clear whether this is the result of low fusion frequencies or the complete absence of any fusion event which could lead to stable prototrophic hybrids. Therefore, it was desirable to increase fusion yields in order to obtain also products of low frequency fusions. In *P. guilliermondii*, variation of the $CaCl_2$ concentration (either 10 to 100 mM), of incubation temperature (between room temperature and 40°C), and of incubation time in PEG (between 1 and 60 min) did not increase fusion yields. However, dimethyl sulfoxide (DMSO) caused an increase as soon it was added to the PEG solution (Table 2). It has been known from other authors that DMSO increases PEG-induced fusions between both animal cells or plant protoplasts (6,7).

Table 1 Fusion yield of some fusion experiments

Parents	Number of protoplasts per ml which were able to regenerate	Fusion yield
799 met-1 nic-2	2.1×10^{6}	1.9×10^{-4}
809 his-1 ade-8	1.9×10^{5}	
799 asn-1 his-7	7.0×10^{2}-1.0×10^{7}	1.2×10^{-3}-9.5×10^{-2}
799 met-1 arg-3	1.0×10^{3}-2.2×10^{7}	
799 met-1 ade-4	6.0×10^{6}	1.7×10^{-6}
799 met-1 arg-3	1.8×10^{7}	
799 asn-1 his-7[a]	1.0×10^{6}	7.6×10^{-4}
799 asn-1 ino-1	1.3×10^{5}	
fp 12-1 met-1[b]	1.0×10^{4}	6.0×10^{-4}
799 asn-1 his-8	1.5×10^{5}	
fp 15-1 asn-1[a,b]	2.7×10^{4}	3.7×10^{-4}
799 met-1 arg-3	1.6×10^{6}	

[a] PEG contained 15 % DMSO
[b] fp 12-1 met-1 is a fusion product of the combination 799 met-1 ade-4 + 799 met-1 arg-3, and fp 15-1 descends from 799 asn-1 his-7 + 799 asn-1 ino-1.

Table 2 Influence of DMSO on the fusion yield in the combination 799 met-1 arg-3 + 799 asn-1 his7

Experiment	Fusion yield PPM	30 % PEG + 100 mM $CaCl_2$	30 % PEG + 100 mM $CaCl_2$ + 15 % DMSO
1	2.5×10^{-5}	1.2×10^{-3}	6.0×10^{-3}
2	0	1.3×10^{-6}	2.2×10^{-5}

Table 3 Spontaneous mitotic segregation of some fusion products

Parents	Fusion products	Segregation frequencies	Markers
809 his-1 ade-3	fp 1-6	0.1×10^{-2}	-
799 met-1 nic-1	fp 1-17	2.1×10^{-2}	ade
	fp 1-61	0.3×10^{-2}	-
799 met-1 arg-3	fp 3-1	0.2×10^{-2}	met arg
799 asn-1 his-7	fp 3-8	0.2×10^{-2}	met
	fp 3-15	0.1×10^{-2}	-
	fp 3-26	0.2×10^{-2}	met arg
	fp 3-33	0.3×10^{-2}	-
	fp 3-51	0.1×10^{-2}	-
	fp 3-70	0.5×10^{-2}	-
799 met-1 ade-4	fp 12-1 met-1	1.5×10^{-2}	met ade arg
799 met-1 arg-3	fp 12-2 met-1	0.2×10^{-2}	-
	fp 12-4 met-1	0.3×10^{-2}	met ade
	fp 12-5 met-1	0.4×10^{-2}	met ade
799 asn-1 his-7	fp 15-1 asn-1	6.7×10^{-2}	asn ino
799 asn-1 ino-1	fp 15-2 asn-1	62.4×10^{-2}	asn ino
fp 12-1 met-1	fp 13-1	1.0×10^{-2}	his
799 asn-1 his-8			met arg
	fp 13-2	0.2×10^{-2}	his asn met
fp 15-1 asn-1			
799 met-1 arg-3	fp 16-1	0.3×10^{-2}	-

Genotypes of the fusion hybrids were investigated by induced mitotic segregation with acriflavine or benomyl as inducing agents. In most cases, the fusion hybrids showed heterozygosity for all parental markers if a sufficient number of segregants could be investigated. Only in the hybrid produced by two subsequent fusions (799 met-1 arg-3 + 799 met-1 ade-4) + 799 asn-1 his-8, the ade-marker was not recovered. In most cases not only parental types but also recombinants were segregated from the hybrids. The recombinants can only arise if karyogamy has occurred in the fusion hybrid. In the fusion combination between 799 met-1 arg-3 and 799 asn-1 his-7 we found preferential mitotic co-segregation of unlinked parental markers, though karyogamy must have occurred (4).

The genetic stability of the prototrophic fusion hybrids was investigated by estimating the spontaneous mitotic segregation (Table 3). In the fusion between the two strains 799 met-1 nic-2 and 809 his-1 ade-8, stable as unstable hybrids arose. In other combinations only stable fusion products could be found. In one fusion between 799 asn-1 his-7 and 799 asn-1 ino-1 we obtained only two fusion products with both of them showing a relatively high spontaneous segregation of asn ino. The hybrid with the higher stability was then fused with a diauxotrophic mutant 799 met-1 arg-3. The resulting fusion hybrids were mitotically stable.

The DNA contents of the fusion hybrids were measured in order to get evidence about their ploidy levels (Table 4). In the products of some fusion experiments, we measured a DNA content which was nearly twice that of the parents, but some fusion hybrids contained only slightly more DNA than their parents. Hybrids with low or high DNA-content can arise in one and the same fusion combination.

Table 4 DNA-contents of fusion products

Parents	DNA-content per cell (fg)	Fusion products	DNA-content per cell (fg)
799 met-1 nic-2	27 ± 3(9)	fp 1-6	41 ± 8 (12)
809 his-1 ade-3	22 ± 2(9)	fp 1-61	28 ± 3 (8)
799 met-1 arg-3	26 ± 3(12)	fp 3-70	44 ± 2 (15)
799 asn-1 his-7	28 ± 6(3)		
799 met-1 arg-3	26 ± 3 (12)	fp 12-1 met-1	48 ± 5(12)
799 met-1 ade-4	32 ± 2(16)		
fp 12-1 met-1	48 ± 5(12)	fp 13-1	48 ± 3(35)
799 asn-1 his-8	34 ± 4 (11)	fp 13-2	58 ± 5(25)

In order to increase the DNA-content of the strain 799 by protoplast fusion, in the first step the two diauxotrophic mutants 799 met-1 arg-3 and 799 met-1 ade-4 were fused. In this way we obtained fusion hybrids auxotrophic for met. One of these hybrids, which contained 83 % of the expected DNA-content, was fused with the diauxotrophic mutant 799 asn-1 his-8. These "triple" hybrids contained only slightly more DNA than the parental fusion product.

DISCUSSION

As in other yeasts, *P. guilliermondii* can be hybridized by protoplast fusion in a parasexual way in order to obtain hybrids with a twofold ploidy level. The method does not lead to stable hybrid cells in each case. The instability is not only limited to fusion products of a fusion between cells of different strains. In combinations of certain mutants of one and the same strain unstable hybrids can occur, whereas, stable hybrids arise out of other combinations.

A lower DNA-content of the hybrids than expected can be the consequence of such instabilities caused by mitotic chromosome loss. However, this is in contrast to the fact that the fusion hybrids with a DNA-content as high as that of the parental strains (i. g. fp 1-61, Table 4) are heterozygous for all the four parental markers. Another possibility for explaining the low DNA-content is fusion of parts of the parental genomes. The enhancement of the DNA content to a threefold level of the parental strains has not been successful until now. It may be that the chosen mutants or fusion combinations cause instabilities within the first mitotic cycles and mitotic chromosome loss leads to the lowering of DNA-content per cell.

SUMMARY

Fusions were performed between and within different strains of *Pichia guilliermonddii* with the intention to enhance the ploidy level. Stable and unstable diploids arose. Stable hybrids contained either nearly the expected DNA-content or only the DNA--content of the parents, though they were heterozygous for all parental markers.

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INTERGENERIC FUSION OF YEAST PROTOPLASTS: *SACCHAROMYCES CEREVISIAE* + *SCHIZOSACCHAROMYCES POMBE*

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Intergeneric hybrid cells prepared by protoplast fusion may divide; however, their further development to true hybrid organisms has not been observed so far (1). For the study of morphogenesis of fusion products, microbial protoplasts seem to be more suitable, as the hybrid organism can be recovered after the fused protoplast has reverted to a cell. A simple technique for protoplast preparation and fusion, availability of auxotrophic strains and powerful selection procedures provide a basis for a large scale experimentation. The first successful results have recently been published (2) but their genetic interpretation is not well understood yet.

In the present experimental work the method of protoplast fusion was applied to obtain hybrids from morphologically distinct yeast strains, ellipsoid *Saccharomyces cerevisiae* and cylindrical *Schizosaccharomyces pombe*, with the aim to study morphological features of the hybrids. The results showed, however, that although the fusion occurred and hybrid protoplasts tended to grow, no convincing evidence was obtained that occasional prototrophic cells were true intergeneric hybrids.

MATERIALS AND METHODS

Strains: Haploid auxotrophic strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used. Their list and combinations are given in Table 1.

Media: Cells were grown in malt-extract medium. Selection medium for protoplasts consisted of Leupold's minimal medium (3) supplemented with 0.7 M mannitol (MM) and 2 % (w/v) agar (MMA).

Protoplast preparation and fusion: Protoplasts were prepared by a routine method (4). The release of *Sch. pombe* protoplasts from cylindrical walls was achieved by washing the digested cells with 0.7 M mannitol, at 37°C , containing 1 % (w/v) glucose. Both types of protoplasts were then washed with 0.7 M mannitol. The fusion suspension contained 5×10^7 protoplasts of each partner; these were mixed thoroughly and centrifuged. The pellets were

then suspended in 2 ml of 35 % (w/v) polyethylene glycol 4000 (PEG) containing 0.1 M $CaCl_2$. After 20 min of incubation at 37°C samples were taken for cytological analysis and regeneration procedures.

Fusion analysis: For regeneration, the treated protoplasts were mixed with MMA and the suspension was poured onto plates. To follow the growth ability of the fused products, the suspension in PEG was streaked on MMA films (4). Samples of PEG-treated protoplasts were also resuspended in MMA medium containing 30 % (w/v) PEG 4000 and incubated in thin layers on plates. For cytological observation, the PEG-treated protoplasts were slowly diluted with MM medium and their smears were stained with Giemsa's reagent. Samples of PEG-treated protoplasts were taken also for preparation of freeze-etched replicas (5).

RESULTS AND DISCUSSION

1. Characteristics of Parent Cells and Protoplasts

The cells of parent strains were of typical species-specific shape, ellipsoid and cylindrical. The stability of genetic markers was sufficiently high; no prototrophic colonies appeared on MMA medium from an inoculum of 10^6 cells.

No marked differences were found between both types of protoplasts. Neither of them grew on the surface of MMA media. *S. cerevisiae* protoplasts increased in volume, apparently due to enlargement of the vacuoles, *Sch. pombe* protoplasts became optically denser as the result of incipient cell wall regeneration. Protoplasts did not produce visible colonies after an inoculum of 10^7 protoplasts was embedded into MMA medium. In media supplemented with required growth substances, about 10 % of *S. cerevisiea* and 20 - 30 % of *Sch. pombe* protoplasts reverted to visible colonies.

Table 1 Strains and their combinations

Saccharomyces cerevisiae 291 trp1 ura4 met4 leu tyr7 α	+	*Schizosaccharomyces pombe* 1137 ade6 his h^-
Saccharomyces cerevisiae 291 trp1 ura4 met4 leu tyr7 α	+	*Schizosaccharomyces pombe* his5-303 h^-
Saccharomyces cerevisiae 9 his α	+	*Schizosaccharomyces pombe* ade7-50 h^-
Saccharomyces cerevisiae 11 ade2 ilv1 trp5 α	+	*Schizosaccharomyces pombe* his5-303 h^-

2. Fusion

Protoplasts of both types were mixed to give nutritional complementation (Table 1).

The addition of PEG to pelleted protoplasts led to a strong agglutination. Upon dilution the aggregates were disrupted into single protoplasts. If the dilution was prolonged up to one hour, some protoplasts remained in close contact as groups of two or three bodies. The same picture was found when the agglutinated protoplasts were transferred on MMA films. Large bodies corresponding to fused protoplasts were not observed. Staining of PEG-treated and then slowly diluted protoplasts showed that about 20 % of protoplasts contained two nuclei, 2 % were trinucleated and also bodies with four nuclei were occasionally found. Control preparations treated in a similar way except PEG, contained 2.3 % (*Saccharomyces cerevisiae*) and 5.7 % (*Schizosaccharomyces pombe*) binuclear protoplasts.

The observation of freeze-fractured aggregates confirmed by close contact of protoplast surfaces. On cross-fractured faces, breaks in the membrane continuity were found (Fig.1). On P faces, particle-free, rounded areas were revealed suggesting that plasma membrane fusion was at least initiated (5). These results, together with the findings of binucleated protoplasts, indicate that the fusion of protoplasts could occur. It seems that it was initiated in PEG and finished later during regeneration in selection medium. It cannot be concluded from cytological analysis whether the binucleated bodies of partly fused protoplasts originated from different partners or from protoplasts of the same type.

3. Cultivation on the Surface of MMA Media

As with the control protoplasts, most of the PEG-treated protoplasts transferred onto the surface of MMA did not grow (Fig.2). Among them, large vacuolated formations appeared after 12-20 h of incubation (Fig. 3). A progressive increase in volume of the cytoplasm was accompanied by the formation of fibrillar wall, which was an indication of active growth. These growing bodies were found in all tested combinations at frequencies ranging from 10^{-4} to 10^{-5}. They were mostly growing out from the aggregates. However, single growing protoplasts were also seen. A slow dilution of PEG-treated protoplast mixtures with MM medium did not decrease the frequency of the vacuolated bodies. These were not found in mixtures of complementary auxotrophs that had not been treated with PEG.

The occurrence of single growing bodies that were in close proximity to other protoplasts suggests that cross-feeding can hardly be responsible for their growth. Similarly, the fact that from the aggregate composed of many bodies only one or two protoplasts were growing can be taken as evidence that lacking nutritional substances were not provided by occasionally lysed protoplasts.

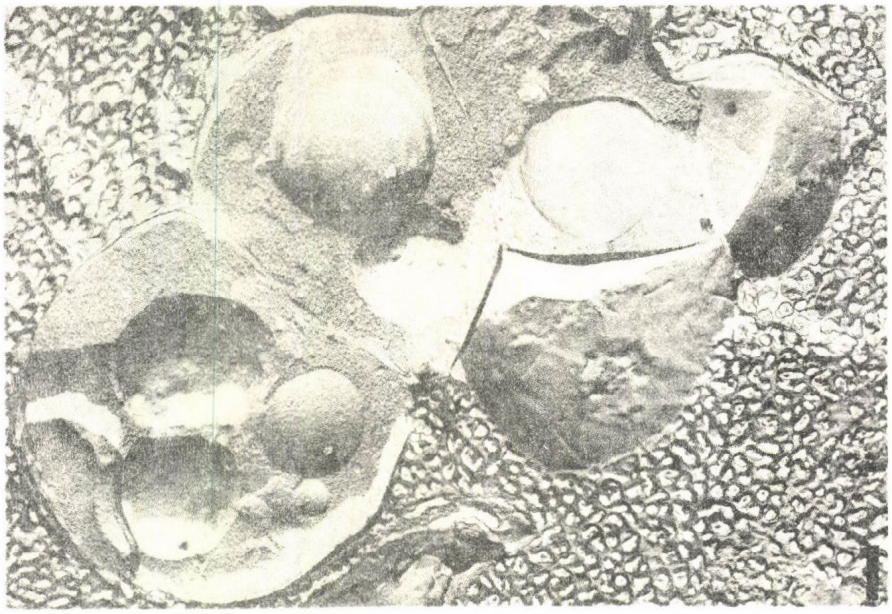

Fig. 1. Freeze-etched protoplast aggregate of *S. cerevisiae* 291 and *Sch. pombe* his after 20 min incubation in PEG. Note the border between protoplasts with a folding of the membranes.

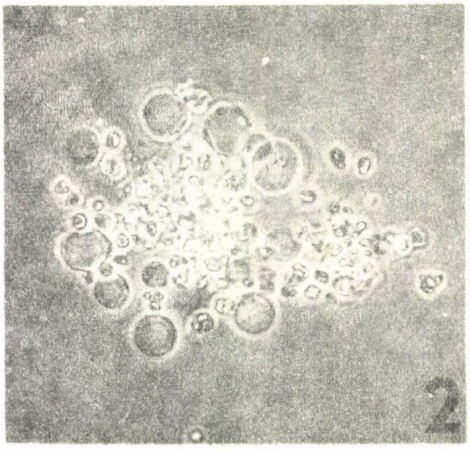

Fig. 2. Non-growing group of PEG-treated protoplasts. Incubation on the surface of MMA at 28°C for three days.

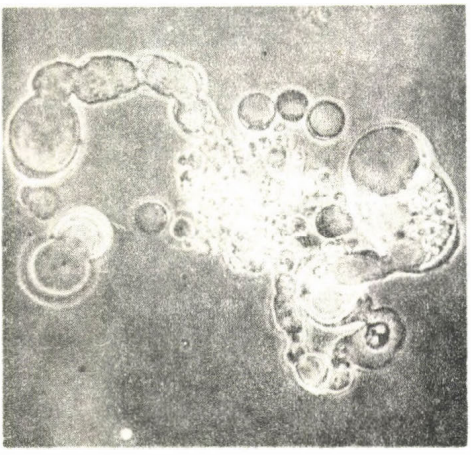

Fig. 3. Two growing vacuolated formations in a group of non-growing protoplasts. Combination of *S. cerevisiae* 291 and *Sch. pombe* 1137.

These facts lead to an assumption that the growing bodies are prototrophic and originate from successfully fused *S.cerevisiae* and *Sch.pombe* protoplasts.

4. Regeneration in MMA and PEG-Containing Media

In three of four combinations tested no reversion of protoplasts to cells was detected, either macroscopically or microscopically. Spherical walled bodies with no proliferation activity could be occasionally found after a week's incubation. After the same incubation period the combination of *S. cerevisiae* 291 with *Sch. pombe* his5-303 gave rise to some small colonies, the frequency of which was 10^{-6} or less. The morphology of the cells was either ellipsoid or cylindrical, as were their parent strains. Their limited propagation capacity did not permit genetical analysis. Because of their occasional occurrence and extremely low frequency these cells cannot be taken simply as prototrophic revertants of fused protoplasts.

In PEG-containing media, which also support cell wall regeneration and reversion to cells, no prototrophic colonies from the four intergeneric combinations were recovered.

The negative results can be explained by the assumption that the complete restoration of morphogenesis (i.e. cell wall regeneration and cell division) requires a certain degree of integration of both partners' genomes, while growth (i.e. triggering of macromolecular synthesis) is ensured by the mere presence of the complementing genomes in a common cytoplasm.

SUMMARY

An attempt was made to produce intergeneric hybrids between two morphologically distinct yeast species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* via their protoplast fusion. Cytological analysis indicated that fusion occurred. The fused protoplasts were able to grow on the surface of selection media. No convincing evidence was obtained that occasional prototrophic cells were true intergeneric hybrids.

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HYBRIDIZATION OF YEAST FROM GENUS *HANSENULA* BY PROTOPLAST FUSION

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Hybridization makes it possible to increase the effectiveness of the selection work with yeasts by constructing new species combining valuable parental properties. Sexual hybridization is now widely used in selection of higher plants and baker's yeast *Saccharomyces cerevisiae*.

Most of the industrial yeast strains producing feed proteins are sexually inactive. That is why the method of protoplast fusion that has been developed intensively in many laboratories for the last few years seems to be very promising for hybridization of such strains. In this communication the results of experiments on protoplast fusion performed for hybridization of methanol-utilizing, non-mating strains of *Hansenula polymorpha* are presented. The method was also developed for other *Hansenula* species.

STRAINS

Three methanol-utilizing strains (ML-3, KT-2, Z) resulted from the screening programme carried out in our laboratory were used. According to their morphological and physiological characteristics these strains may be refereed to as *Hansenula polymorpha*, although all of them lack the important taxonomic property of sporulation. The stock strain of *Hansenula polymorpha* (de Morais et Maia) VKM 1397 received from the Institute of Microbiology of Acad. Sci. USSR was also used. It is a homothallic, sporogenous haploid with very low methanol-utilizing capacity. Strains of *Hansenula bimundalis* and *Hansenula anomala* were obtained from the Institute of Technical Chemistry (Leipzig, GDR).

INDUCTION OF AUXOTROPHIC STRAINS

Nitrosoguanidine (NG) was usedas a mutagen. Stationary cultures were treated with NG (200 μg/ml) in phosphate buffer (pH 6.5) at 28^{o}C for 25-30 min, then were plated on complete medium (yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l, agar 20 g/l), and auxotrophs were isolated after replica plating on minimal medium (Rider's medium with 20 g/l glucose, 20 g/l agar, and vitamins). The frequency of auxotroph appearance was ap-

proximately 1-4 % for most of the strains, but for KT-2 and Z it was only several hundredths of a per cent. Most of KT-2 and Z auxotrophs were leaky and reversible. One may suppose that ploidy of the strains KT-2 and Z was more than haploid.

After several passages stable auxotrophs were selected. These auxotrophs were treated with nitrosoguanidine again and double auxotrophic mutants were isolated and used in the following experiments.

PROTOPLAST FORMATION

Protoplasts were formed by the method reported by Zaikina and Lapotyshkina (1). Mutant cells were grown on supplemented minimal medium for 16 h., washed twice with 0.04 M β-mercaptoethanol in the same buffer for 20 min at 28°C (for *H. bimundalis* 0.1 M β-mercaptoethanol was used). Then cells were treated with snail enzyme (2 mg/ml) in the same buffer supplemented with 0.6 M KCl as a stabilizer. After 40-70 min of enzyme treatment (depending on species) more than 99 % of the cells were converted into protoplasts. Initially ellipsoid yeast cells may be seen under the microscope; these are converted into spherical forms. For the quantitative estimation of protoplasts we used the proportion of cells which did not keep their viability after dilution in distilled water (only protoplasts of the strain VKM 1397 were not sensitive enough to osmotic shock). Protoplasts were collected and washed twice by centrifugation. Regeneration capacity was checked by growth in agar layer. Complete medium was used with 0.6 M KCl or 0.4 M $CaCl_2$ as a stabilizer. The level of regeneration was higher with 0.4 M $CaCl_2$ than with 0.6 M KCl, but some of the mutants were unable to form colonies (e.g. ML-3 met lys) in the presence of this stabilizer.

PROTOPLAST FUSION

The fusion experiments were carried out as described by Sipiczki and Ferenczy (2). Freshly prepared protoplast suspensions of the two mutants, each containing $2\text{-}5 \times 10^7$ protoplasts, were mixed and centrifuged. The supernatant was then removed and 1 ml 35 % polyethylene glycol (molecular weight 6,000) in 0.01 M $CaCl_2$ was added to the pellet; tubes were gently shaken and incubated for 20 min at 28°C. Then samples of the mixture were spread onto the surface of minimal medium containing 0.6 M KCl (or 0.4 M $CaCl_2$) and covered with 3 ml of the same medium. After 5-7 days prototroph colonies may be observed. In control experiments, protoplasts of each parental strain were plated and mixtures of intact cells were treated in the same way. No prototrophic colonies were observed. The fusion frequency was calculated by comparing the number of colonies growing on minimal medium to the number of regenerated protoplasts of the minority parent.

The fusion frequency of *Hansenula polymorpha* protoplasts was 10^{-4} - 6×10^{-3}. There was no remarkable difference in the fusion frequency when double mutants of the same or different methanol-utilizing strains were used.

The above fusion techniques were also used for obtaining intra-species hybrids in *Hansenula anomala* (lys ura x his leu) and *H. bimundalis* (ade lys x arg cys). Prototrophs were formed with the frequency 6.7×10^{-5}-1.1×10^{-4}, with the best results being obtained with *H. anomala* in 0.4 M $CaCl_2$ and with *H. bimundalis* in 0.6 M KCl. Some experiments on interspecies protoplast fusion were carried out with methanol-utilizing strains and *H. anomala* or *H. bimundalis*, but we have never observed any prototrophic growth.

CHARACTERISTICS OF FUSION PRODUCTS

Prototrophic forms maintained their ability to grow on minimal medium through many passages. After plating on the complete medium and replica plating on the minimal medium, clones seemed to be relatively stable, since spontaneous segregation of auxotrophs did not exceed 1-2 % for most of the fused forms (Table 1). Only in the combination, KT-2 x VKM 1397, single unstable clones arose in which the proportion of auxotrophs reached 30 %.

The cells of fusion products were larger than those of parental strains. Methanol-utilizing strains ML-3 , KT-2, and Z were asporogenous. Also, their fusion products were not able to form spores on any of the sporulation media tested. Only prototrophs which arose from the fusion of methanol-utilizing strains and VKM 1397 formed asci on malt extract agar medium (ME). Among them, cultures may be found with rare (less than 0.1 %) or few asci as well as abundantly sporulated cultures (up to 70 % asci); colonies on ME varied in color from slightly pigmented to bright pink. All the later prototroph cultures were capable of active methanol utilization, while the parental strain, VKM 1397, displayed only weak methanol-utilizing capacity.

HYBRID NATURE OF THE FUSION PRODUCTS

To determine if the prototrophs were the result of cell and nuclei fusion one needs to estimate the presence in the prototrophs of parental auxotrophic markers. We have analyzed auxotrophs segregating from prototroph fusion products spontaneously, or after UV-light treatment (known to induce mitotic recombination). In some experiments the cells were treated with parafluorophenylalanine (pFA) or were irradiated with γ-rays for the induction of aneuploids. The results are presented in Table 1.

Prototrophs tested may be seen to harbour all the parental auxotrophic markers. The presence of recombinant (non-parental) auxotrophic markers suggested that fusion of the nuclei was necessary for recombination. The data also showed that the best result in the detection of heterozygous auxotrophic markers was obtained with γ-ray treatment. It has been shown previously that γ-irradiation induces aneuploidy in the diploid yeast *Pichia pinus* by chromosome loss (3).

Table 1 Spontaneous and induced mitotic segregation of the fusion products

Strains	Parental markers	Spontaneous auxotrophs in single clones (%)	Number of auxotrophs tested	Segregating auxotrophic markers
ML-3 x x ML-3	met his x x ade cys	0.9 1.4 1.4	60, spontan	57 met, 2 cys, 1 his
			98, γ-rays	70 met, 8 his, 4 cys 9 ade, 1 met his 2 his ade, 1 his cys, 1 met cys, 2 met his ade cys
			14, pFA	12 met, 1 his, 1 ade met
			43, UV-light	40 met, 2 cys, 1 ade
ML-3x xVKM 1397	met his x x arg leu	0.25 0.15 0.3 1.2 0.3 0.2	23, spontan.	19 met, 3 leu, 1 leu met
			117, UV-light	100met, 11 leu, 6 his
ML-3xZ	ade leu x x met ura	0.1 0.2	2, spontan	2 ade
			18, UV-light	8 ade, 2 met, 1 met ura, 1 leu met, 4 ade met ura (2-unknown)
Zx x VKM 1397	arg leu x x met ura	0.4 0.4 0.9 0.7 0.7	4, spontan	2 met, 2 met ura
			12, UV-light	2 met, 2 met ura, 1 arg ura, 6 arg met ura 1 arg leu met ura
KT-2x xVKM 1397	ade met x x arg leu	2.5 9.5 0.3 30.5 1.1 12.7 6.3	122, spontan	113 leu, 5 arg, 1 ade, 3 met
			154, UV-light	94 leu, 4 arg, 1 ade 50 met, 2 arg leu 3 leu met
ZxZ	met ura x x ade leu	2.3 1.0	12, spontan,	2 ade, 10 met ura
			55, UV-light	2 met, 2 leu, 4 ade, 43 met ura, 3 met ade, 1 ade leu

DNA CONTENT

Some months after performing the above experiments, the content of DNA per cell was determined in all initial parental strains and in some hybrids. All of these strains were put in stock and from time to time they were transferred to a fresh nutrient medium. The results are presented in Table 2.

Table 2 The DNA content per cell in different yeast strains

Strains	DNA content (10^{-12} g/cell)
ML-3	14
VKM 1397	15
KT-2	30
Z	29
VKM 1397 x VKM 1397	27
ML-3 x ML-3	29
ML-3 x VKM 1397	29
Z x VKM 1397	30
Z x Z	34

It may be seen that parental strains ML-3 and VKM 1397 had the lowest DNA content per cell and seemed to be haploid, while KT-2 and Z strains had a DNA content twofold that of ML-3 or VKM 1397 and seemed to be diploid.

Thus, after fusion of ML-3 x ML-3 or ML-3 x VKM 1397 we obtained diplois, after fusion of ML-3 x Z or VKM 1397 x Z - triploid strains, and after fusion of Z x Z - tetraploid strains. According to the DNA content, hybrids resulting from fusion of ML-3 and VKM 1397 seemed to be diploid as well as those from LM-3 x x Z, VKM 1397 x Z and Z x Z fusions. Perhaps, triploids and tetraploids were transformed into diploids during storage.

CONCLUSION

By protoplast fusion of haploid and diploid non-mating strains of *Hansenula polymorpha* intra- and interstrain hybrids were obtained with different ploidy. The diploid hybrids were stable while triploid and tetraploid hybrids were unstable. The instability of triploid and tetraploid states of *H. polymorpha* was expressed as an increased frequency of auxotrophic marker segregation and decrease in DNA content per cell during the storage.

ACKNOWLEDGEMENT

We thank Dr. A.V. Temina for determination of DNA contents in yeast strains.

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A STUDY ON PROTOPLAST FUSION AND PARASEXUAL HYBRIDIZATION OF ALCANE UTILIZING YEASTS

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ABSTRACT

Optimum conditions for preparation and regeneration of protoplasts from *Saccharomycopsis lipolytica*, *Candida guilliermondii*, *Pichia pinus* and *Candida boidinii* have been studied and protoplasts of *Sm. lipolytica* and *C. guilliermondii* were fused. Protoplasts of the investigated strains have been shown to be able to regenerate on the surface of solid media, even under limited growth conditions. A large number of intra- and intergeneric fusion products was studied cytologically and genetically. The fusion frequency was higher in intraspecific fusions (0.1-0.6 %) than in intergeneric ones (0.1 %) and depends strongly on the state of protoplasts and their ability to regenerate. Intraspecific hybrid cells do not differ significantly from parental cells in size, but they do so in DNA content. All fusion products have been shown to contain only one nucleus. Intergeneric fusion products segregate after few passages in contrast to intraspecific hybrids.

During the last decade the protoplast technique has been shown to be a very effective tool for biochemical and substructural investigations of fungal and yeast cells overcoming the barrier represented by the thick cell wall. Moreover, the use of protoplasts opened the way to hybridization experiments and genetic studies with plant, fungal and yeast cells lacking sexual processes, even between distantly related strains or organisms. In recent years an increasing number of papers dealing with the study of protoplast fusion for genetical purposes has been published and standard techniques for preparation, regeneration and fusion of protoplasts from different mycelial fungi and yeasts were developed in certain laboratories (1-5). In particular, interspecific and intergeneric hybrids have been obtained from *Sm. lipolytica* and *C. tropicalis* (6,7).

To study and improve regeneration of protoplasts to complete cells in different yeasts to be used for fusion or transformation experiments, protoplasts of the alcane utilizing yeasts *Saccharomycopsis lipolytica*, *Candida guilliermondii* and the

methanol utilizing yeasts *Candida boidinii* and *Pichia pinus* obtained by snail enzyme treatment were plated on solid minimal or complete media containing different osmotic stabilizers. In contrast to *Saccharomyces cerevisiae* protoplasts of these yeasts show good cell wall regeneration on the surface of YEPD- and minimal media with glucose as carbon source or even on mineral salt medium without any carbon source, stabilized with sorbitol (1.4 M), $CaCl_2$ or KCl (0.4 M).

By means of polyethylene glycol (MW 6000, 40 %) protoplasts of diauxotrophic mutant strains of the alcane utilizing yeast *Saccharomycopsis* (*Candida*) *lipolytica*, belonging to opposite and like mating types have been shown to fuse and regenerate on solid agar media.

TABLE 1 Combination of partners in intraspecific fusion experiments with *Saccharomycopsis lipolytica*

Mating types / Genetic markers	Fusion frequency %
A (ilv, lys) + A (met, ade)	0.1
A (ilv, lys) + A (arg, ade)	0.1
B (arg, ade) + B (arg, leu)	0.2
A (ilv, lys) + B (arg, ade)	0.1
A (ilv, lys) + B (arg, leu)	0.6

Fusion frequencies up to 0.6 % have been achieved depending on protoplast titer but no on mating type. As shown by scanning electron microscopy, the condition of protoplasts and preservation of their viability strongly influence the fusion frequency of protoplasts or the regeneration ability of fusion products (Fig. 1). By means of scanning electron microscopy it was demonstrated, that PEG treated protoplasts were closely associated. After washing, impressions resulting from adjoining protoplasts could be demonstrated. The surface of protoplasts appears to be shrunken and covered with small particles, probably PEG (Fig. 4 b-d).

About 150 prototrophic hybrid colonies which have been shown to be stable after many passages were isolated. Fusion products of different mating type combinations: A + A, A + B, B + B were selected for cytological and genetical characterization. Significant differences in cell size of parental cells and cells from hybrids as described by other workers (3,5,9) have not been recognized as demonstrated by microscopic measurements.

Some homozygous hybrid cells tested have been shown to be able to conjugate (AA x BB) and sporulate on corresponding media. The DNA content of fusion products was higher than in parents; however, no exact correlation between content of DNA and state

TABLE 2 DNA content in cell of parental strains, fusion products (FP) and sexually produced diploids (D) in *Sm. lypolytica*

Parents/fusion products	Designation of strains, mating types	Content of DNA fg/per cell
parent (n)	S 4-2 (B)	54.7 ± 1.1
"	S 26/10 (A)	53.0 ± 1.7
"	S 59 (A)	58.1 ± 0.1
"	S 64 (B)	70.4 ± 0.2
"	S 113 (A)	64.8 ± 1.1
FP 1	S 64 + 4-2 (B+B)	132.4 ± 1.1
FP 2	"	165.3 ± 1.4
FP 3	"	155.0 ± 1.4
FP 26	S 26/10 + S 59 (A+A)	115 ± 0.8
FP 27	" "	129.4 ± 1.1
FP 28	" "	161.0 ± 1.4
FP 65	S 26/10 + S 64 (A+B)	102.6 ± 1.1
FP 80	" "	131.0 ± 1.4
FP 81	" "	123.1 ± 0.6
D 38 (2n)	S 26/10 x S 4-2	112.3 ± 0.2

of ploidy was established. DNA was estimated by the DABA method (10).

In intergeneric fusion experiments the following strains have been used:

Saccharomycopsis lipolytica m.t. A (arg, ade), m.t. B (ade,arg).

Candida guilliermondii (H), (ade, his), (ade, ura)

The fusion frequency in these experiments has been shown to be only 0.01 %.

The nuclear state of hybrid cells is of general interest. On regenerating protoplasts, multipolar budding can be observed suggesting the presence of more than one nucleus or the successive division of a single nucleus and following formation of more

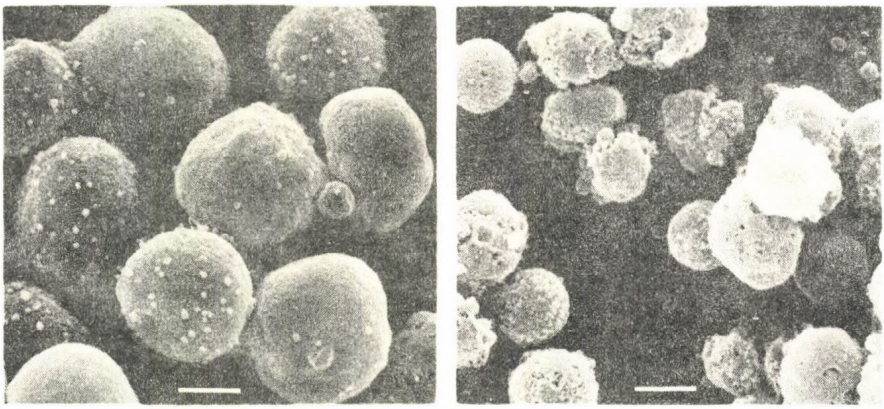

Fig. 1. Differences in preservation of protoplasts. Scanning electron micrograph. Bar represents 1 µm.

Fig. 2 Regeneration of a fusion product in *Sm. lypolytica* and formation of more than one daughter cell from the regenerated mother cell. Magnification 1800 x.

than one bud. This suggestion is based on the observation that during protoplast formation of budding yeast cells binucleate protoplasts can be formed, which originate in the common release of protoplasts by the mother and daughter cells both with a nucleus. Therefore, in a protoplast fusion experiment , a large number of binucleate protoplasts might participate in the fusion process. This must be considered when the nuclear state of fusion products or the segregation pattern is estimated and interpreted. Therefore, in our investigations the nuclear state of different hybrid cells has been studied carefully.

However, in all hybrid cells isolated from prototrophic colonies resulting from interspecific and intergeneric fusion experiments only one nucleus was shown to be present by phase contrast microscopy in living cells using optimum of refractometric indices (11) and by fluorescent microscopy after staining with acridine orange, suggesting formation of synkaryons in fusion products (Fig. 3). In contrast to intraspecific fusion products which have been shown to be stable in prototrophy and morphology for some months, the intergeneric fusion products after few passages revealed increasing morphological similarities to one or the other initial strains used in fusion experiments and the appearance of auxotrophic clones. In a preliminary study the segregation of genetical markers has been investigated by random spore analysis and by haploidization using MBC, Benomyl and p-fluorophenylalanine. MBC has been shown to be most effective. The isolation of stable hybrids or recombinants by protoplast fusion or transformation not only provide new genetic tools but also offers the possibility of constructing new microorganisms.

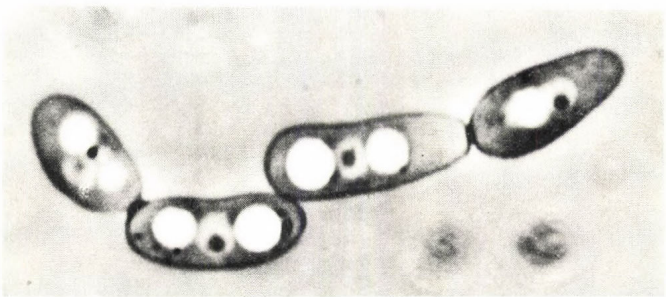

Fig. 3 Intraspecific hybrid cells from *Sm. lipolytica* containing only one nucleus and a nucleolus.
(Gelatine n_D = 1,378)
Magnification 2400 x.

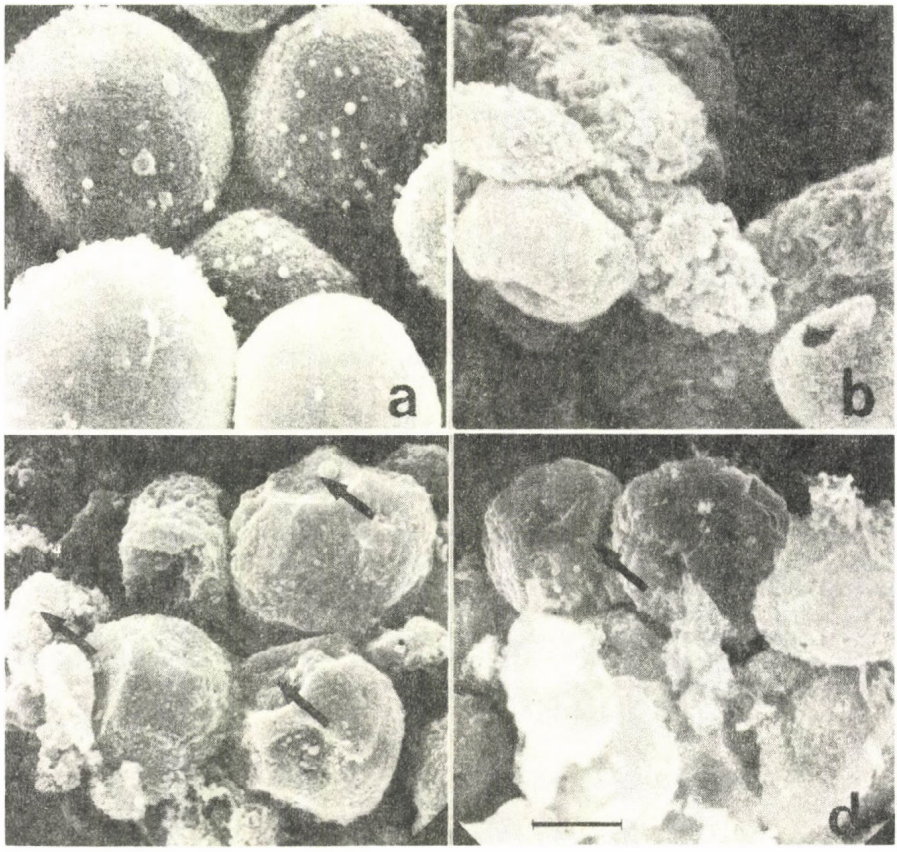

Fig. 4 Yeast protoplasts before (a), during (b) and after (c,d) treatment with PEG. Impressions (arrows) resulting from adjoining protoplasts during PEG-treatment are visible.
Bar represents 1 µm.

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CONSTRUCTION OF POLYSACCHARIDE-DEGRADING BREWING YEASTS BY PROTOPLAST FUSION

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Brewing yeasts are strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis having many properties which distinguish them from the rather more typical laboratory strains of these species. These properties have probably arisen by quasi-natural selection over the many millions of generations in which they have been used in the brewing industry. Brewing yeasts are polyploid or aneuploid and largely asporogenous - the few spores which are produced being of low viability. Such anomalies make the study of these yeasts impossible by conventional genetic techniques, and the advent of protoplast fusion has offered workers in this field a valuable new method with which to embark on strain-improvement programmes.

Fermentation with conventional brewing yeasts produces a beer in which remains 20 mg carbohydrate/ml. This is exclusively non-fermentable polysaccharide material of heterogeneous size and structure conveniently termed dextrin. Saccharomyces diastaticus is able to utilize at least some of this dextrin, but unfortunately also produces unacceptable flavour compounds in beer. The aim of the experiments reported here was to produce a hybrid between Saccharomyces carlsbergensis and Saccharomyces diastaticus which would produce an acceptably flavoured beer containing a reduced concentration of dextrin.

Two major problems had to be overcome before such a hybrid could be obtained. Firstly, because the Sacch.carlsbergensis strain SC was polyploid it obviously proved extremely difficult to introduce reliable markers into it. Limited success was achieved to this end with the isolation of cadmium resistant and adenine requiring derivatives, but the problem was better overcome by exploiting the natural vitamin requirement of the strain. It has an absolute requirement for biotin for growth. Isolation of marked derivatives of Sacch.diastaticus 625 proved simple since strains could be sporulated on potassium acetate. Several multiply marked strains were obtained by sequential mutagenesis. The characteristics of strains used in fusions are listed in Table 1.

TABLE 1. CHARACTERISTICS OF STRAINS USED IN FUSIONS.

Strain	Characteristics	Source
SC	bio	Bass reference collection
SC-Cd30.4	bio Cd^r	U.V. mutagenesis of SC
SC-A1	bio ade	U.V. & nystatin enrichment of SC
625	bio pan	Brewing Research Foundation Yeast Collection
625-111	bio pan ade met thr	U.V. of 625 after sporulation
625-112	bio pan trp ade ile cys	U.V. of 625 after sporulation
L164R	bio pan	Bass reference collection

Symbols bio pan and Cd^r specify biotin requirement, pantothenate requirement and resistance to 1mM $CdSO_4$ respectively.

The second major problem to be overcome was the regeneration and selection of hybrids from the fusion. The regeneration frequency of Sacch.diastaticus protoplasts on dextrin (rather than on glucose) varied with strain from a negligable level to a few percent. Regeneration was extremely slow, typically taking 3 - 4 weeks and even after this time colonies were extremely small. In addition picking of colonies for analysis from within agar gels was both inefficient and tedious. To alleviate these problems several modifications to the basic regeneration system were investigated. Some of these are shown in Table 2. Although washing in sorbitol supplemented with amyloglucosidase markedly stimulated regeneration of Sacch.diastaticus protoplasts, problems were envisaged in counterselecting against the SC parent in crosses, since this strain could form small colonies on the products of dextrin hydrolysis by the enzyme. 30% gelatin (1) was therefore adopted for regeneration in fusions since it allowed quantitative recovery of regenerated protoplasts for a two stage selection of hybrids.

TABLE 2. REGENERATION FREQUENCIES OF SACCH. DIASTATICUS PROTOPLASTS IN DIFFERENT REGENERATION SYSTEMS.

The standard procedure was to wash helicase-prepared protoplasts three times in 1.2M sorbitol, spread on 3% agar plates and then overlay with the same medium.

Regeneration System	% Regen.	Comments
Standard 3% agar. Glucose as C-source	10	Control
Dextrin as C-source	3.4	Very slow. Small colonies.
Dextrin + Tween 80 (10mg/ml) + ergosterol (25µg/ml)	4.0	Faster regeneration. Larger colonies
Wash in glucose (10mg/ml 1.2M sorbitol)	0	Complete lysis on washing.
Wash in amyloglucosidase (1.0nl/ml sorbitol)	10	Regeneration comparable to glucose.
Add cellophane sheet between protoplasts and top layer.	2.8	Fast regeneration. Colonies sampled by lifting cellophane.
30% gelatin. Glucose as C-source	N.D.	Micro-colonies after 24h. Easily removed by dissolving gelatin.

Unless otherwise stated carbon-source for regeneration was dextrin (10mg/ml).

N.D. : Not determined.

The first diastaticus-carlsbergensis hybrids were obtained from a fusion of SC-A1 and 625-111. To improve the yield of hybrids the diastatic strain in this cross was U.V. irradiated to 99.99% kill before protoplasting as suggested by Hopwood and Wright (2). After fusion protoplasts were regenerated for 24h. in gelatin media selecting against 625-111 only, released from the gel by dissolution in distilled water and counterselection against SC-A1 applied by inoculation into media containing dextrin as sole carbon-source. The procedure is set out in detail in Figure 1.

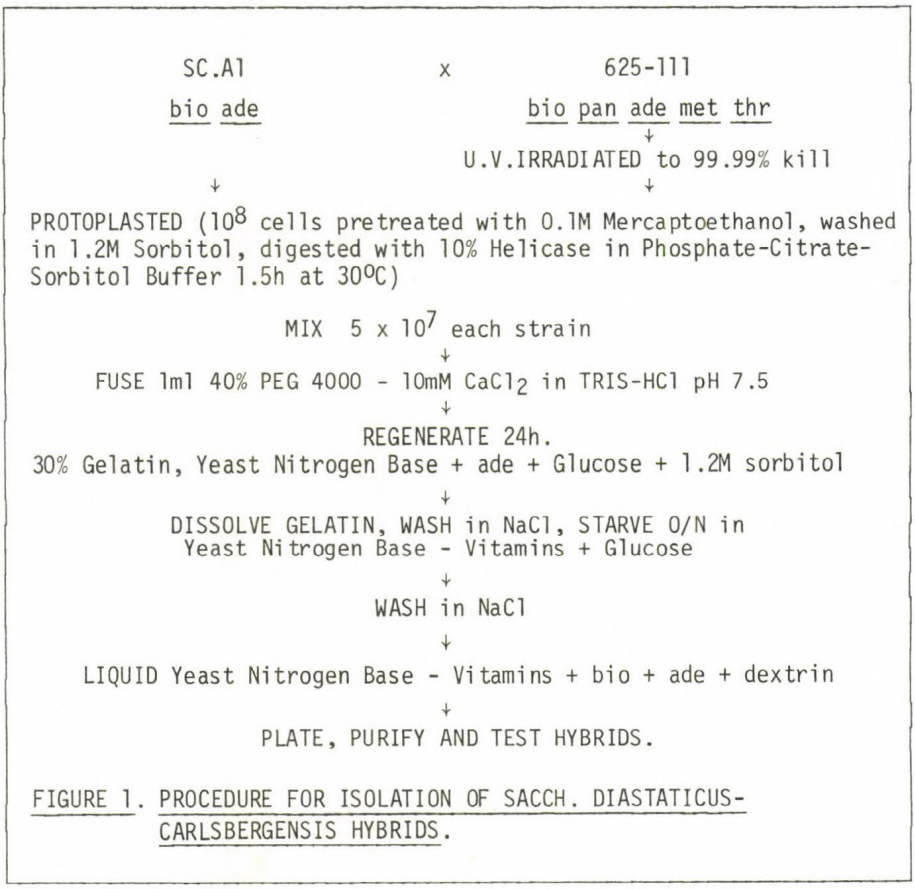
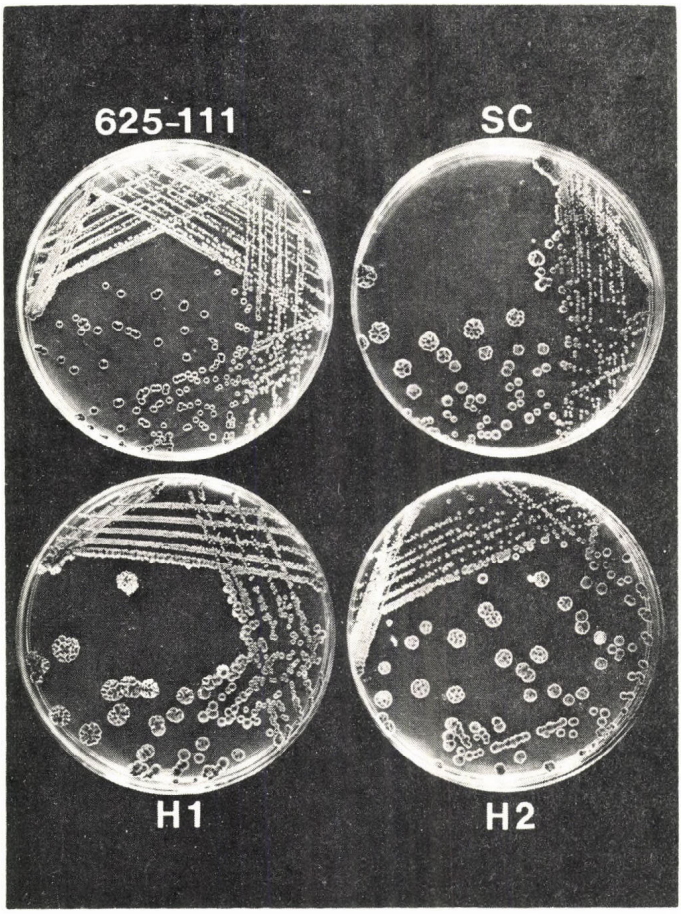


FIGURE 1. PROCEDURE FOR ISOLATION OF SACCH. DIASTATICUS-CARLSBERGENSIS HYBRIDS.

The products of this fusion were morphologically indistinguishable from SC-A1 on WLN agar (Plate 1), but were able to utilize dextrin. They had an absolute requirement for biotin and retained a leaky requirement for adenine. They did not require methionine or threonine.

PLATE 1. APPEARANCE OF PARENTAL & HYBRID STRAINS ON WALLESTEIN AGAR.



625-111: Sacch.diastaticus parent. SC: Sacch.carlsbergensis parent. H_1 and H_2: Independent hybrids.

Their fermentation profiles were of the SC rather than 625 type except that the residual carbohydrate concentration in the beer was lower than with SC. These results are consistent with the interpretation that the products of the fusion are true diastaticus-carlsbergensis hybrids. Further experiments are in progress to verify this conclusion.

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THE USE OF MITOCHONDRIAL MUTANTS IN THE ISOLATION OF HYBRIDS OBTAINED BY FUSION OF PROTOPLASTS OF BREWING YEASTS

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The method of protoplast fusion has been considered for several years as a method of obtaining new hybrids of brewer's and distiller's yeasts. While successful fusion of protoplasts from *Saccharomyces* species has been obtained by several workers, the yeast strains employed have normally been laboratory strains, carrying various auxotrophic markers, which made isolation of the hybrid strains relatively easy. Brewing yeasts, being mostly diploid or of higher ploidy, present difficulties to the geneticist attempting to introduce auxotrophic markers into producing strains, since such auxotrophs are frequently of considerably reduced vigor in the first place, and are, in addition, unstable. Thus, the production of strains carrying adequate markers to permit identification and isolation of the hybrids formed on fusion of the protoplasts presents special problems for investigators using brewing yeasts.

Fewer difficulties are encountered in the introduction of mitochondrial rather than chromosomal markers into brewing yeasts. Both petite mutations and mutations to antibiotic resistance can be readily obtained. Strains resistant to various heavy metals (Mn, Cu, Co and Ni) can also be obtained without difficulty, though the precise location of these markers in brewing yeasts is not always known. In this paper we report the use of mitochondrial markers to isolate hybrids obtained by fusion of protoplasts from some brewing and distiller's yeasts, and present a protocol for use by investigators wishing to use this method to obtain similar hybrids.

MATERIALS AND METHODS

Yeast strains. The strains used were YS 2968, a distiller's yeast, OK17, OK20, L1 and DICH, brewing yeasts from various sources; and NCYC 625, a strain of *Saccharomyces diastaticus*, obtained through Bass-Charrington Limited. The chloramphenicol and erythromycin-resistant strains of NCYC 625 and the erythromycin-resistant strain of DICH were made by selection on antibiotic-containing media after treatment in a medium containing 8 mM $MnCl_2$ according to the method of Putrament et al. (1). NCYC 625 (*S. diastaticus*) normally grows on starch as a sole carbon

TABLE 1 Protocol for protoplast formation and fusion

Grow cells to 10^8 ml (exponential phase)
↓
Spin down 2 ml
↓
Incubate 10 min at 37°C with 4 ml of pretreatment solution[1]
↓
Wash once with 1.2 M sorbitol solution
↓
Resuspend in buffered protoplasting soln[2]
↓
Add 0.1-0.15 ml of snail enzyme
↓
Incubate at 37°C until protoplasting is complete
↓
Spin down (500 xg)
↓
Wash twice in 0.4 M $CaCl_2$
→
Resuspend in 0.4 M $CaCl_2$ and count
↓
Mix equal numbers of protoplasts to a total of 10^8 protoplasts
↓
Spin down, 500 xg, and remove supernatant
↓
Add 2 ml of 35 % PEG soln and resuspend, gently
↓
Incubate 10 min at 37°C
↓
Dilute 10x and 100x in 35 % PEG solution
↓
Dilute into melted OSA[3] at 44°C and pour over OSY agar[4-6] and any other media desired. Use plates prewarmed to 37°C. Incubate at 30°C.

[1]Pretreatment solution contains tris-buffer, pH 7.8, 0.2 M; EDTA, 0.02 M; β-mercaptoethanol, 0.1 M, and sorbitol, 1.2 M.

[2]Protoplasting solution contains phosphate-citrate buffer, pH 5.8, and sorbitol, 1.2 M.

[3]OSA = 1.5 % agar + 0.6 M KCl.

[4]OSY = 1.5 / agar + 1.0 % glucose + 0.5 % yeast extract + 0.6 M KCl.

[5]OSSG = 1.5 % agar + 2 % glycerol + 2.0 % soluble starch + + 0.6 M KCl + 0.5 % yeast extract.

[6]OSS = 1.5 % agar + 2 % soluble starch + 0.5 % yeast extract+ + 0.6 M KCl.

source, and the erythromycin-resistant strain of DICH also produces zones of clearing when grown on a starch-containing medium. The petite forms of these three antibiotic-resistant strains were made and then used in the fusion process.

Genetic Techniques

Sporulation was done in a liquid medium (McClary's) inoculated (1:10) with a 48-hr culture grown in YEP-glucose medium at 30°C, and incubated for 3 days, with aeration, at 28-30°C. Sporulating cells were recovered and washed twice with sterile water, and stored in the refrigerator until required.

Dissections were done after digestion of the ascus wall with snail digestive juice, using a flat-ended needle and a Singer micromanipulator, by the method of Johnston and Mortimer (2).

Protoplast formation was done according to a protocol provided by the Department of Genetics, University of Washington. Protoplast fusion was carried out according to the protocol used by Ferenczy, for *Candida tropicalis* (3).

The methods used are summarized in Table 1.

The protoplasting was carried out by using snail digestive juice.

After fusion and plating on OSY agar, the plates were incubated at 30°C until colonies began to appear.

At this point, several methods were used for initial selection of hybrids. Since strains NCYC 625 and DICH-E^r both gave zones of clearing on starch-containing media, the colonies showing such zones on OSSG and OSS media, containing starch + glycerol and starch only as C sources, the colonies showing zones, after refrigeration of the OSSG and OSS media for several days, were picked and tested further. In addition, colonies were picked at random from regenerants on OSY plates and tested for starch hydrolysis, growth on YEP-glycerol and resistance to chloramphenicol and erythromycin. Finally, regenerants on OSY plates were replica-plated by velvet pad to CAP and erythromycin media, and colonies growing on these media were also picked and tested.

RESULTS AND DISCUSSION

As can be seen from Table 2, as would be expceted, more respiratory-competent strains (growth on glycerol) which showed starch-clearing were obtained from the OSSG and OSS media than from random selection from colonies on the OSY plates. Most of the strains obtained were resistant to chloramphenicol and erythromycin, but in crosses with YS 2968 this was not regarded as significant, since the latter strain proved to have some resistance to these antibiotics normally.

In crosses of 625-C^r(p) and 625E^r(p) with OK17 and OK20, which were sensitive to these antibiotics, regenerants arising on OSY agar were replicated to CAP and erythromycin-containing me-

TABLE 2 Strains isolated from fusion of brewing yeasts

Strains fused	Number of isolates	No.clearing starch	Growth on YEPG	Spo	C^r	E^r
625-C^r(p)x L1	8	4	8(w)	2	0	-
625-C^r(p)x2968	24	10	24	9	24	-
DICH-E^r(p)x2968	32	12	32	-	-	32

dia, and colonies growing on these media were isolated. On these media, both parent strains were effectively eliminated, and only hybrids obtained by fusion would be expected to grow. While these hybrids have not been completely characterized, all gave clearing zones on starch media, as expected (data not shown).

The progeny of some of the lines were isolated after sporulation and dissection and tested further, in particular for starch clearing.

TABLE 3 Segregants from sporulating strains isolated from hybrids

Strain	Total spores	Viable spores	Large colonies	Tetrads	Starch clearing tested	+	-
625-C^r(p) x 2968							
-2A	44	33	17	2	24	16	8
-2E	44	44	22	11	23	4	19
-3C	40	28	15	3	21	12	9
-3D	40	37	21	8	25	12	13
DICH-E^r(P) x 2968							
10-2E	52	50	25	11	24	9	15
11C	48	48	24	12	26	12	14

As can be seen from Table 3, segregation for starch clearing, on a random-spore basis, was approximately 2:2, as would be expected if this character is under the control of a single nuclear gene. However, the behavior of the single-spore clones obtained from these strains reduces the accuracy of these data to some extent at least. While total spore viability was high, as determined by their initial growth on the dissection medium,

segregation between normal and pin-point colonies was 2:2 in almost all asci tested, and while the normal colonies grew even more vigorously than is usually observed, not all of the pin-point colonies gave rise to viable clones. The exact segregation ratios are thus in some doubt as a result. However, only in the case of strain 2E of the cross 625-C^r(p) x 2968 was a relatively low ratio of positive to negative clones obtained in the starch-clearing test. Where complete tetrads were obtained, 2:2 segregation was frequent, as expected.

The protocol suggested for the use of mitochondrial markers in the hybridization of brewing yeast strains is shown in Table 4.

TABLE 4 Suggested procedure for hybridizing brewing yeasts

(1) Select strains to be fused

(2) Test strains for antibiotic resistance and select an antibiotic to which the strains are sensitive

(3) Make mutants resistant to the antibiotic(s) selected

(4) Make petite mutants from the antibiotic-resistant strains

(5) Test the petites for retention of the antibiotic-resistance marker, by crossing to a suitable auxotrophic strain using RD-auxotrophic method

(6) Test the petites also for stability of the protoplasts to eliminate fragile strains

(7) Carry out the fusion process, mixing protoplasts from RD strains and antibiotic-sensitive RC strains

(8) Regenerate the protoplasts and fusion products on OSY and OSYG agar, and any other media which may seem appropriate

(9) Replica plate from the OSY and OSYG media to antibiotic-containing media

(10) Check the strains thus obtained for other markers, if any, especially those from the RD strain, since this parental strain can be completely eliminated by testing on YEPG.

Particular attention should be paid to the strain which is to be the petite partner in the fusion procedure, and it should be tested by conventional hybridization with a sensitive laboratory strain, preferably a mating diploid, to ensure that the mutation to antibiotic resistance is rescuable in the cross. For instance, the mutation to chloramphenicol and erythromycin resistance in NCYC 625 is retained in the petite mutant, while that to oligomycin resistance is not, in any of the strains tested so far. In addition, the conditions of protoplasting should be tested to determine the stability of the protoplast from the petite strain, which may be more sensitive than those from the normal strains, or even too fragile for use. The nature of the pretreatment solution may affect the stability of the protoplasts, and in

particular, the concentration of β-mercaptoethanol may have to be reduced for protoplasting the petite form.

Thus, mitochondrial markers such as mutations to antibiotic resistance can be used successfully in the isolation of hybrids obtained by the fusion of protoplasts of brewing yeasts. The hybrids thus obtained may not, of course, be suitable for immediate use as brewing yeasts per se, since Stewart (personal communication) has stated that some hybrids obtained in this way have undesirable characteristics not found in either parent strain. This may be related to the quantitative as opposed to qualitative nature of the characters of a strain defined as a good brewing yeast, where over- and under-production of the same compound may be equally undesirable. Hence, complete control over the performance of brewing yeast strains may only be obtained when the metabolism of the minor compounds produced by brewing yeasts, and its genetic control are properly understood. Protoplast fusion, followed by the production and study of recombinants from the hybrids thus obtained, may be added to classical genetic techniques as a powerful tool in the elucidation of these mechanisms.

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REGENERATION OF PROTOPLASTS

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One of the general and fundamental properties of all living systems is the ability to repair injury made to their structures and functions. Protoplast regeneration is no more than an expression of this ability. It is now well established that all walled cells, disregarding their evolutionary history, are potentially capable of regenerating the cell wall when this has been artificially removed. There is also an analogy to this process in nature, in naked zoospores, where the *de novo* formation of the cell wall must be regarded as a physiological process. It is known, however, that cell wall regeneration is, in many situations, not only a simple renewal of a missing structure but is a process connected with profound changes which, in the protoplast, induce a set of SOS repair mechanisms. The reversion of nucleated subprotoplasts is evidently something more than a simple cell wall regeneration. In protoplasts where several cell cycles have taken place between conversion and cell wall regeneration, the reversion is much more complicated than a simple renewal of normal cytokinesis. Therefore, the process of protoplast regeneration cannot be regarded as being identical with the process of cell wall regeneration or with the protoplast reversion. Besides controlling cell wall renewal, the process of reversion involves a number of various regulatory mechanisms responsible for cell integrity.

Cell wall regeneration and protoplast reversion in different cells have been reviewed several times (1-7). Many review articles can also be found in the Proceedings of the International Symposia on Protoplasts held in 1965, 1968, 1972 and 1975 (8-11). The aim of this communication is (i) to show that the first observations on these phenomena date back to the early history of experimental cytology, (ii) to present a very general view of the problem, accentuating the events common to all reverting protoplasts rather than drawing attention to differences specific for various kinds of cells, and (iii) to outline some perspectives for reverting protoplasts to be used as a model system.

SOME REMARKS ON THE HISTORY

The first experiments with cell regeneration were performed by Dujardin in 1838 and by Naegeli six years later. Both came to the conclusion that separated parts of cells cannot continue to live. Some of the earliest observations that focussed on cell wall regeneration are listed in the following table:

Vaucheria	- Hofmeister 1867	- negative results
Penicillium, Mucor	- Brefeld 1872	
Vaucheria	- Hanstein 1873 - Strassburger 1876	
Mucor	- van Tieghem 1875	
Syphonocladium	- Schmitz 1879	
Vaucheria	- Sachs 1882	- importance of the nucleus
Helodea	- Klebs 1887 - Townsend 1897	- "Kernstoffe"
Phycomyces	- Burgeff 1914, 1917	- fusion

All observations and experiments listed above have been carried out on single cells and the techniques used for protoplast liberation have been limited to mechanical dissection, plasmolysis and a combination of both.

A new era opened for cell wall regeneration studies with the advent of techniques for large-scale preparation of protoplasts. This was first achieved in bacteria but had no importance at that time since the regeneration ability of bacterial protoplasts - in contrast to that of sphaeroplasts (12) - was recognized much later. A considerable number of yeast protoplasts were prepared by controlled mechanical disruption of cells (13) and by controlled autolysis of the cell walls (14). The first observations on the behaviour of protoplasts, including their mass ability to regenerate to new viable cells, were published in 1955 (14, 15). After Eddy and Williamson developed the excellent routine technique for protoplast preparation using lytic enzymes of *Helix pomatia*, numbers of papers appeared describing protoplast liberation and cell wall regeneration in a wide veriety of cells. The most important findings are summarized below:

Yeasts	- Nečas 1955, 1956	- autolytic, gelatine
	Rost and Venner, 1965	- snail enzymes
Moulds	- Emerson and Emerson 1958	
	Aguire and Villanueva 1962	- strepzyme

Green plants	- Pojnar et al. 1967	
	Power and Cocking 1970	- after fusion
Bacteria	- Landman et al. 1968	- gelatine
	Schaeffer et al. 1976	- after fusion
	Fodor and Alföldi 1976	
Green algae	- Gabriel 1970	
Blue green algae	- ?	

Although this survey is far from being complete it shows that all types of cells, including bacteria (16, 17), fungi (3, 5, 6, 18), eukaryotic algae (19) and green plants (20, 21) behave in the same way when regenerating their walls or reverting to cells. However, the list is still incomplete because even though protoplasts have been prepared in prokaryotic algae (12) no reversion has been achieved so far, probably because suitable environmental conditions promoting cell wall regeneration have not been found.

THREE MAIN EVENTS OF PROTOPLAST REGENERATION

The process called protoplast regeneration could be divided into three events (Fig.1)(i) regeneration of the missing cell wall; (ii) reversion, when a new generation of cells - revertants - arise from a walled protoplast; (iii) the event in protoplast regeneration which can be defined as the growth phase. Removal of the cell wall during protoplasting does not interfere with continuation of the cell cycle (in those cells that are not irreversibly arrested in G_0 phase of the cycle). Consequently, there may be one or several doublings of the cell mass. This is most pronounced in protoplasts of the cells that have a short generation time in relation to the cell wall regeneration period, in other words, where there is a considerable lag between protoplasting and reversion.

1. Cell Wall Regeneration

As mentioned above, the ability to regenerate a new cell wall is inherent to all cells. This does not necessarily mean that this potential is realized in any environmental conditions. In protoplasts of certain cell types (e.g. budding yeast, bacteria, and probably blue green algae) a new complete cell wall is not assembled in routinely used cultivation media without providing certain physical environmental conditions. The most frequently used regeneration techniques are: embedding in high percentage gelatine (23) or 2 % agar (24, 25), suspending in 30% polyethyl-

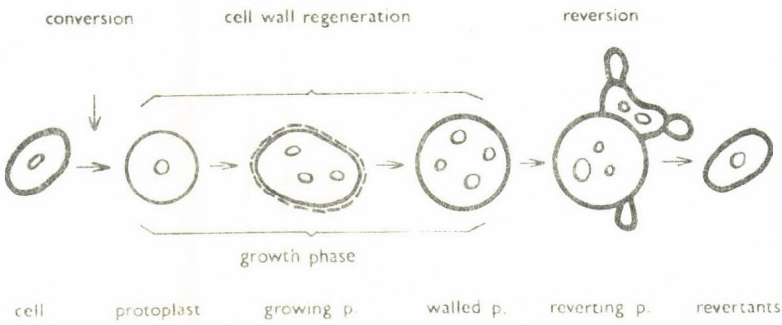

Fig. 1. Schematic representation of the main events of protoplast regeneration.

ene glycol (26) and accumulating bacterial protoplasts in a pellet or on membrane filters (16). Also the chemical composition of nutrient medium appears to be important for successful wall regeneration (21).

A question was much discussed as to whether the assembly of cell wall components does require a supramolecular template, for instance in the form of the original cell wall remnants (27), or if it is a *de novo* formation. It seems clear now that the formation is a *de novo* process, since it has been proved by both electron and fluorescent microscopy that the observed bodies are indeed true protoplasts and not sphaeroplasts.

Although direct evidence has not been obtained it is highly probable that the biosynthesis of all cell wall components is not fundamentally influenced during the process of conversion. It remains open whether in protoplasts maintained for a long time only in osmotic stabilizers some kind of synchronization does not occur as the result of a metabolic shift-down. The first cell wall structure appears on the protoplast surface after a lag period which lasts, depending on the cell type, from 15 min. to several hours or even days. In all eukaryotic protoplasts, including those occurring naturally (28, 29), the first wall structures formed are always the fibrils (3, 21, 30, 31) composed either of crystalline glucan, chitin or cellulose according to the chemical composition of the original cell wall. After a short period, the fibrillar nets are masked by the amorphous matrix. This delay in the formation of the amorphous component may be explained by the fact that a larger amount of matrix-

-building units must be accumulated before they can be assembled into a supramolecular structure.

Experimental data indicate that in eukaryotes the biogenesis of the fibrillar component and that of the amorphous component are independent processes that are controlled by different mechanisms. Inhibition of both protein and RNA synthesis promptly stops the formation of the wall matrix (32, 33) whereas, the formation of the fibrillar component may continue. This suggests that the enzymes polymerizing the corresponding macromolecules of the fibrillar skeleton have a very low turnover. Similarly, in *Saccharomyces cerevisiae* the analysis of cell cycle mutants has revealed that there is no restriction point in the whole cell cycle as for the formation of fibrils (34).

The length of the cell wall regeneration period varies from about 30 min. in moulds to several days in green plants, being related roughly to the normal generation time of the corresponding cells. In addition, it depends strongly on environmental conditions; the "regeneration" media accelerate cell wall synthesis considerably and increase also the proportion of protoplasts capable of accomplishing the regeneration of the cell wall.

The regenerated walls often show marked differences in both chemical composition and submicroscopic structure when compared with the cell walls of intact cells (3,35,36,37,38). Also the three-dimensional architecture (topology) of regenerated walls usually cannot be compared with the genetically determined specific cell shape, but more or less follows the actual protoplast shape determined by the mode of protoplast growth. In this respect the regenerated cell wall behaves as a morphogenetically passive structure (3).

The completion of cell wall regeneration is usually manifested by the initiation of reversion. However, some situations are known where the wall regeneration is not followed by reversion at all (18,38,39).

It remains to be stressed that the cell wall regeneration can be experimentally inhibited, totally or partially, by different means independently of the protoplast growth (32,39,40,41,42). This seems to be important particularly for future experimentation provided that relatively specific inhibitors for different cell wall components or different defective mutants are found.

2. Protoplast Growth

During the growth period, the protoplasts increase in volume due to both macromolecular syntheses and reproduction of all cell organelles. The course of the cell cycle is apparently not affected by protoplasting, and growing protoplasts may undergo mitosis without cytokinesis (19,43,44). The number of cell cycles completed during the growth period is indicated approximately by the number of nuclei (if no protoplast fusion or pas-

sive division occur). There is, however, little experimental evidence to show whether a quantitative relationship exists between the other cell organelles in the protoplasts maintained as in the normal cell cycle: in other words, whether the growing protoplast is a multiple copy of a single cell. Some data indicate (45,46) that in growing yeast protoplasts the quantitative relationship may be markedly altered (such as the number of mitochondria and Golgi bodies, and the extent of endoplasmic reticulum area per volume unit).

The morphology of protoplast growth depends both on physical forces of the cultivation environment and on the wall structures that are gradually formed on the protoplast surface. The latter influence the growth as physical factors rather than a morphogenetically active biostructure. It may also be expected that the organization of microtubules and that of non-muscle actomyosin (microfilaments) will cooperate in determining the shape of growing protoplasts. However, experimental evidence supporting this assumption is still lacking.

The growth period lasts until a new cell wall is assembled. Thus, its relative duration depends on the readiness of a protoplast for cell wall renewal. Results of experiments with temporal inhibition of wall regeneration provide corroborating evidence for this view (39).

On the other hand, the period of protoplast growth is not unlimited even in conditions where cell wall regeneration is continuously inhibited in some way. All protoplasts that have not regenerated their cell walls die after a shorter or longer period. All experiments designed to achieve unlimited existence of a cell in the protoplast state (i.e. without the cell wall) have been unsuccessful . The only exceptions are stable L-forms of a protoplast type in bacteria (47). In this case, however, multiplication of L-forms can hardly be compared with regular cell division. There is little experimental evidence to explain why the naked cell is definitely limited in its survival. It can be only speculated that (i) repeated cell cycles without cytokinesis may lead to a certain unbalanced state of some quantitative relationships, which is incompatible with some functions in growing protoplasts (e.g. unsuitable surface/volume ratio). (ii) The presence of the cell wall is responsible for the feedback control of cellular processes occurring in and/or on the plasma membrane. These feedback signals may be out of operation only for a limited period of the existence of the system. In any case, further work on this problem that will eventually result in developing a method for cultivating a protoplasmic mass not organized in individual cells will be of great importance in many aspects of cell biology.

3. Protoplast Reversion

A series of events leading to the restitution of a cell identical with the original entity can be regarded as proper reversion. Its onset coincides with the start of the first cytokinesis in

walled protoplasts. The restitution of the normal genetically determined state, however, may be a step-by-step process requiring several cell cycles, i.e. several generations of revertants.

The initiation of cytokinesis is one of the most interesting aspects of protoplast reversion. In any kind of protoplast it is invariably associated with wall regeneration. The new cell wall, no matter how defective or morphogenetically passive, evidently produces signals which are recognized by the system controlling the cytokinesis. Recognizing the nature of these signals should be one of the most important goals in protoplast research and would greatly contribute to the understanding of general mechanisms of cell morphogenesis.

The morphology of reversion varies widely and depends principally on the mode of the cytokinetic mechanism of the corresponding cell. Thus, in *Saccharomyces cerevisiae*, which divides by budding, protoplasts revert through cytokinesis resembling budding; in *Schizosaccharomyces*, which divides by fission, the first cytokinesis in reverting walled protoplasts is characterized by formation of septa, etc. Many aberrations of cytokinesis, found especially during reversion of multinucleated protoplasts, demonstrate that the control of cytokinesis is heavily disturbed. It remains open to speculation whether these alterations are caused primarily by some disorder of the cytoskeletal system of microtubules and microfilaments or by the fact that the regenerated cell walls are largely abnormal in their chemical composition and structure.

It should be added that the revertants differing in biochemical and morphological properties from the original cells were isolated in *Saccharomyces cerevisiae*. These revertants remained stable for thousands of generations (48).

REVERTING PROTOPLASTS AS A MODEL SYSTEM

The primary aim of the study of protoplast regeneration is to discerne the reparative capacity of a cell and to understand the mechanisms controlling the repair processes leading to the maintenance of cell integrity. At the same time, reverting protoplasts provide a unique model system for studying many other cellular processes. At least some of them should be mentioned. The reparative cell wall formation can be separated into two steps, i.e. in formation of fibrillar and of amorphous components. Thus, it is possible to analyze both events separately, starting with the expression of the genes involved and ending with the assembly of the final structures. This approach allows one to recognize pathways of morphogenic information, including the role of self-assembly, at a very general level (49, 50). Reverting protoplasts offer an opportunity to study the morphogenic role of the cell wall in the mechanism of cytokinesis, in the determination of dimorphism, in cell differentiation, etc. This model system has been little used in analysing the relation-

ships between events of the cell cycle, such as interdependence of cell mass doubling and of the initiation of karyokinesis (especially in protoplasts containing more than one nucleus), and last but not least in recognizing the signals that start the cytokinesis.

Reversion of protoplasts is a necessary condition for obtaining viable somatic hybrids by protoplast fusion. It can also be expected that the protoplast state of a cell followed by reversion will offer many opportunities for cell engineering, such as transferring biologically important macromolecules enveloped with liposome membrane or transferring cell organelles derived from one cell to any other cell.

The first historic period covering the first decades of this century showed that any cell is in principle capable of regenerating its cell wall. The second period closed in this decade is characterized by finding that protoplasts of all cells of prokaryotes and eukaryotes are able to revert to cells with full viability, and by determining the most important events of this process. The third period, already started, may bring into fruit the prospects mentioned above, i.e. the use of regenerating protoplasts as a model system for solving many basic problems in the field of cell biology.

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SURFACE ULTRASTRUCTURE OF THE YEAST PROTOPLAST

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ABSTRACT

It has become possible to observe the surface structure of a plasma membrane closely by the freeze-etching technique. We have applied the plasma polymerization technique to study the surface structure of yeast protoplasts. The polystyren replicas were prepared on the surface of the yeast protoplast. The replica films were observed using a transmission electron microscope. This replica film has higher resolution than that of metal replica. The plasma polymerization technique makes it possible to observe the surface ultrastructure of yeast protoplasts without high vacuum damage.

The yeast cell envelope can be largely classified into a cell wall and a plasma membrane. Concurrent with research on the cell wall structure, studies have been made on the plasma membrane. By the development of electron microscopy, it has clarified the existence of plasma membrane, and also revealed that they have unit membrane structure (2,3). A surface view of the plasma membrane of the yeast protoplast was investigated with the freeze-etching technique by many investigators (4,7,9,11). This technique clarified a three-dimensional replica of yeast cells clearly demonstrating the fine structure of yeast plasma membrane. According to this technique, the surface structure of the yeast protoplast could be classified into the invagination and the hexagonally arranged particles which was confirmed by Moor et al. (7). Recently, they reconfirmed the results that such a structure is normal for the yeast plasma membrane structure by ultrahigh vacuum (10^{-9} Torr) freeze-fracturing apparatus (1,6).

On the other hand, Tanaka et al. (9) have made a preliminary study with a glow discharge replica device for obtaining a single stage replica. Applying this new technique, we have studied the surface ultrastructure of yeast protoplasts. This technique also makes it possible to observe the three-dimensional structure of the yeast protoplast.

MATERIAL AND METHODS

Saccharomyces cerevisiae FH4C was inoculated into YEPD (yeast extract, 1 %; peptone, 2 %; dextrose, 2 %) medium. Cells were grown with vigorous aeration for 16 hours at 27°C, washed once with 0.15 M phosphate buffer (pH 7.5), then resuspended in 10 ml of this buffer to which was added 0.5 mg Zymolyase 60,000 (Kirin Brewery, Japan)(5) and 0.8 M sorbitol. The cells were then incubated at 27°C with gentle shaking for one hour, after which they were centrifuged at 2,500 rpm for 5 min. The centrifugate was then gently washed twice with phosphate buffer containing 0.8 M sorbitol to remove most of the enzyme. The preparation was found to contain at least 90 per cent protoplasts.

For electron microscopy, the protoplasts were fixed in 3 per cent glutaraldehyde fixative for 30 min. at room temperature. The specimens were dehydrated with 50 and 100 per cent ethanol for 30 min each. After dehydration, the protoplasts were transferred to propylene oxide. The rough surface of rock candy was used to check the various conditions of glow discharge that may influence the qualities of replica films. The specimen was placed in the glow discharge to fix the polymerized styrene monomer vapour which had been deposited directly on the surface of the rock candy in high energy plasma phase. By this procedure, a rigid polystyrene replica thin film could be obtained. These specimens could be obtained without high vacuum damage when temperature of the surface over the specimens was approximately 20°C during discharge. The most suitable thickness of replica film obtained by this technique was about 100 Å. This replica film was removed, floated on a solution of rock candy, and then picked up on the grid. The replica was observed using the transmission electron microscope.

RESULTS AND DISCUSSION

The surface structure of yeast protoplast was revealed by electron microscopy using a replica film of plasma polymerization in the polystyrene. The polystyrene membrane was deposited on the surface of protoplasts. This is a new replica film, which gave the fine structure and the three-dimensional image by transmission electron microscopy. This image came from a scattering effect of the plasma by glow discharge. Therefore, we could expect a high resolution replica from this replica film of the surface structure in yeast protoplasts or spheroplasts. It is possible to see the shape of the invagination during their cell cycle or whether some structural remnants of the original cell wall are present on their surface.

The fine structure of crystals on the rock salt surface was confirmed by using replica thin film (12). This replica has higher resolution that that of metal replica film. Moreover, three-dimensional images could also be obtained by using uni-

formly thin films. Our experimental results are as follows: Figures 1, 2 and 3 show replica images of yeast cells showing bud scar and birth scars. This also has higher resolution than that of scanning electron microscopy. Figures 4 and 5 show replica images of yeast protoplasts by using the plasma polymerization technique, and the invagination is clearly visible as a groove-like (at arrows) structure. The outer surface of plasma membrane reveals a concave surface of the invagination (Figs. 6, 7 and 8). The size of the invagination is different on each cell; some are short, and others have many branches, especially stationary phase cells such as yeast cakes (4). The structure of the membrane invagination in protoplasts is much different to that of freeze-etched intact cells (4). In the case of the freeze-etched intact cells, we could not clearly identify a groove-like structure.

By the technique of plasma polymerization, we have observed some special part which we assume to be the site of bud formation but we have no evidence to support this assumption. However, during the preparation of protoplasts, bud scars are extremely resistant to attack by lytic enzymes such as snail enzyme or Zymolyase (5). If there are the remnants of an original bud scar with the protoplasts, it could be observed by means of the electron microscope using the plasma polymerization technique. Figure 10 shows the remnant of an original bud scar of a protoplast. Also, it is known that there are endoplasmic reticulum vesicles under the bud scar and also lytic enzyme localized in this part. Moor reported (8) that freeze-etched plasma membrane shows an early stage of bud formation and spherical invagination. We have found the similar structure on the surface of protoplasts (Fig. 9). Nickerson (10) mentioned that, in order to produce budding, there should be a concentration of the appropriate enzyme at the site of bud formation and a local weakening in the cell wall. Therefore, we assume that the part spherical invagination of the plasma membrane will be the mechanically weakening place. According to this idea, we assumed that it would be easy to find a concave shape under the low vacuum condition (Fig. 11 and 12). There is some relationship between the bud scar and the spherical invagination of plasma membrane which is observed by plasma polymerization technique. The remnants of the original cell wall were not present on their surface.

A three dimensional image of the plasma membrane can be observed by means of the plasma polymerization technique. As a result of this an analysis of the surface structure of yeast protoplasts is possible. The preliminary data presented does not enable us to state with certainity the origin of protoplast membrane invaginations.

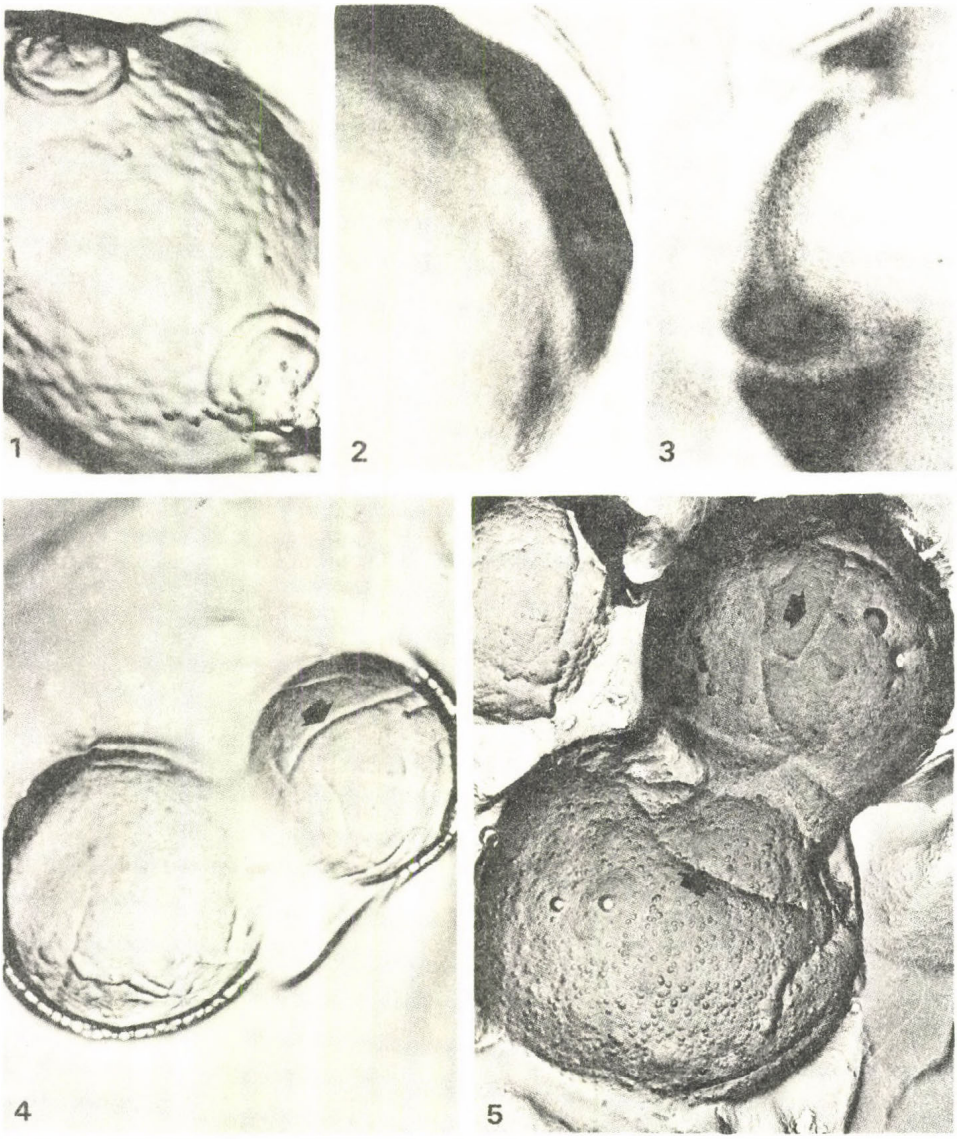
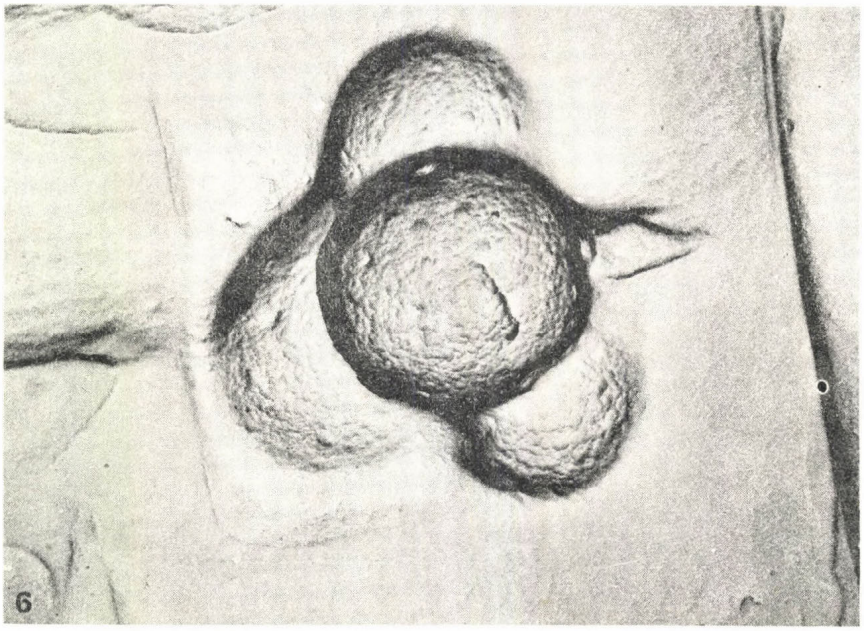


Figure 1. Electron micrograph of a bud scar in surface of protoplast by the plasma polymerization technique. x 20,000. Figure 2. Electron micrograph showing the birth scar region. x 35,000. Figure 3. Electron micrograph showing the bud scar region. x 35,000. Figures 4 and 5. Electron micrographs of the groove-like invaginations (at arrows). x 10,000 and 15,000.



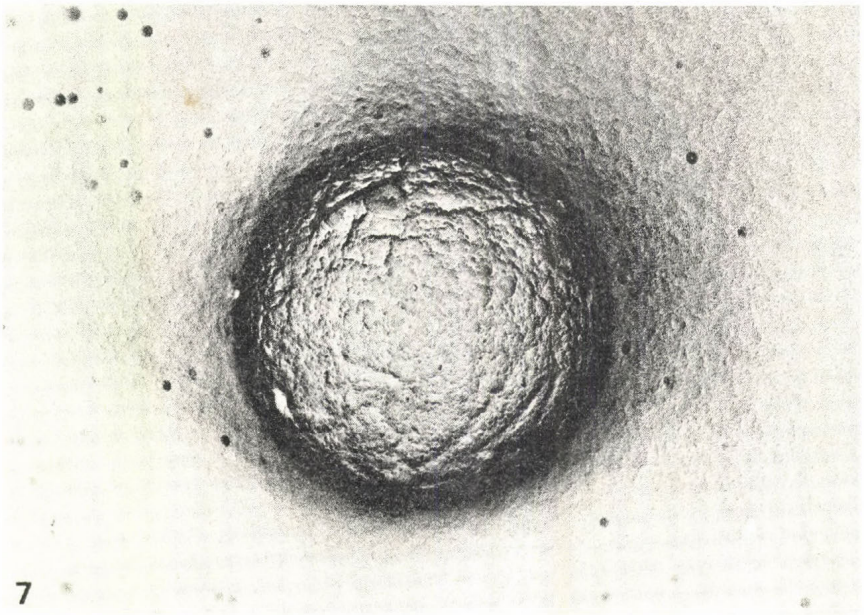
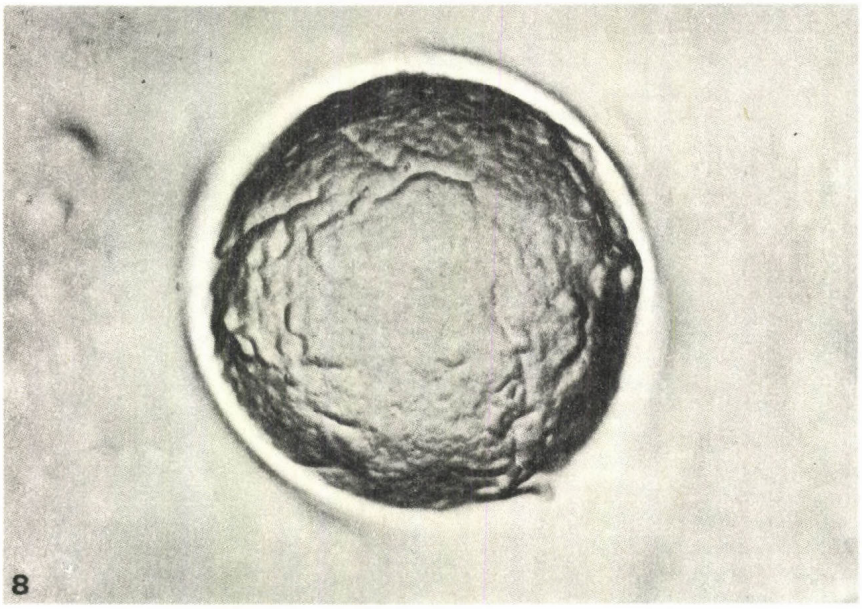


Figure 6. Electron micrograph of four protoplasts in surface view by plasma polymerization technique. x 15,000.
Figure 7. Electron micrograph of protoplast by the plasma polymerization technique. x 15,000.



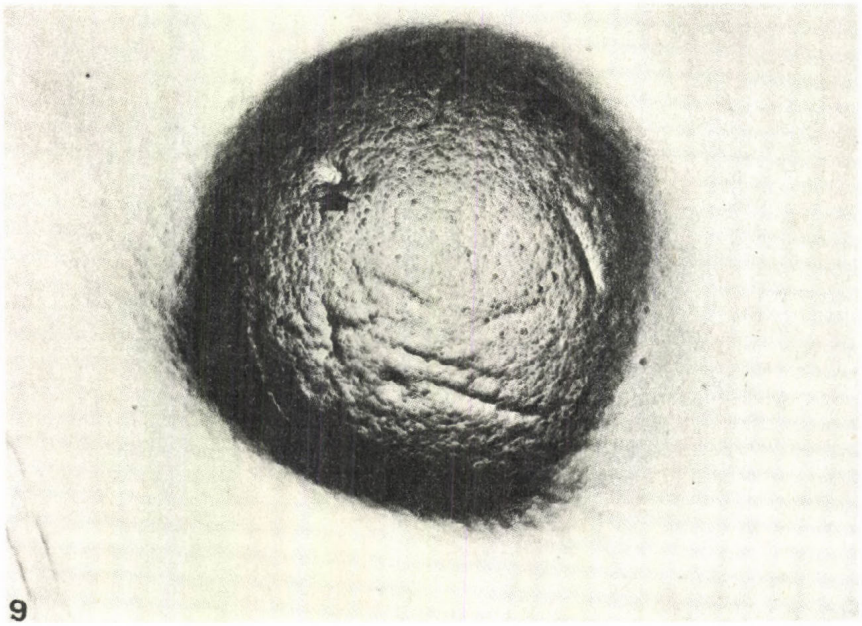
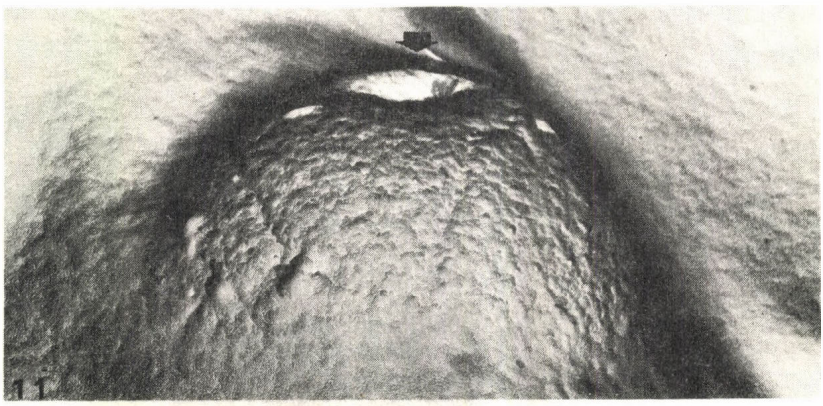


Figure 8. Electron micrograph of a protoplast in surface view of replica film using the plasma polymerization technique. x 15,000.
Figure 9. Electron micrograph of a protoplast in surface view of the spherical invagination (at arrow). x 15,000.



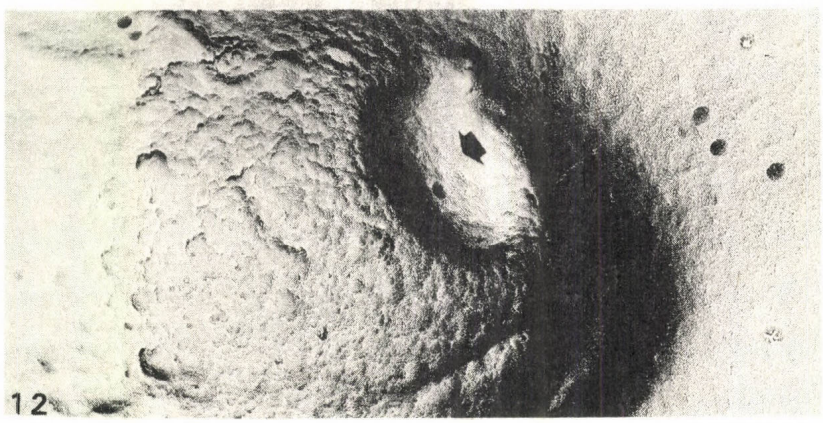


Figure10. Electron micrograph shows the remnant of original bud scar of protoplast. x 20,000.
Figures 11 and 12. Electron micrographs show the concave shape under low vacuum condition. x 20,000.

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A RETROSPECTIVE AND CURRENT VIEW ON ENDOGENOUS β-GLUCANASES IN YEAST

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INTRODUCTION

Because of the importance of β-linked glucans as major structural components of yeast cell walls (1, 2), it appears logical that yeast β-glucanases are directly involved in cellular processes, such as cell expansion, budding, conjugation, and in the lysis of ascus walls of some species. All of these processes probably require a partial breakdown of existing glucan molecules as well as synthesis of new structural glucan. Because yeast glucan is the major polysaccharide responsible for the rigidity and tensile strength of the yeast envelope, the chemistry of this component will be reviewed briefly. Further details are given in references (1, 2).

Alkali-insoluble glucan. This is the principal component responsible for the strength of the yeast cell wall. It is obtained in partially purified form by alkali extraction of purified baker's yeast cell walls (3% NaOH at 75°C). This residue must then be extracted repeatedly with 0.5 M acetic acid at 90°C to extract a β-1, 6-linked glucan component that constitutes about 15% of the crude glucan. The purified residue was shown by Manners and co-workers (3) to be a branched β-1, 3-glucan containing about 3% of β-1, 6-glucosidic interchain linkages. Its molecular weight was about 240,000 and its degree of polymerization approximately 1500. Enzymatic evidence suggests that the chemical composition of this type of glucan varies in different species of yeast (7). The formation of a highly insoluble polymer with a considerable degree of rigidity is thought to be caused by the linking together of β-1, 3-linked chains of glucose residues by hydrogen bonding and/or other forces of attraction. The demonstration by Kopecka et al. (4) of a microfibrillar network as an inner layer of the cell wall supports the idea of aggregation of the unit molecules by association of portions of chains from different molecules to form a continuous structure surrounding the cell.

The β-1, 6-linked glucan component that is extracted by acetic acid is water soluble and highly branched with a degree of polymerization of about 130-140. In baker's yeast glucan this component contains about 19% of β-1, 3-linkages which may serve both as inter-residue and interchain linkages (5). In alkali-insoluble glucan preparations from other species of yeast, the proportion of the β-1, 6-glucan component may be significantly greater than 15% as in baker's yeast (6, 7). Although the function

of this glucan in the cell wall is not known, it has been postulated that it acts as a filling material or plasticizer within the relatively rigid β-1, 3-glucan component (6), possibly by preventing excessive hydrogen bonding.

Alkali-soluble glucan. Several authors have reported the presence of alkali-soluble glucan in yeast cell walls. Fleet and Manners (8) extracted it from baker's yeast cell walls with alkali under nitrogen in the cold. After neutralization, the glucan precipitated as a gel-like material and could be washed. It represented approximately 20% of the cell wall. Its structure appears similar to that of the alkali-insoluble glucan except for a significant proportion of β-1, 6-linked glucose residues inserted into the β-1, 3-linked chains, which may be responsible for the increased solubility in alkali. The presence of some mannose residues in this glucan suggests that it may function as a bonding material between the exterior mannan layer and the interior alkali-insoluble glucan layer.

α-1, 3-Glucan in yeast cell walls. Glucans of this type (also named pseudonigeran) are essentially linear molecules that play an important structural role in the cell walls of many fungi but are found in relatively few species of yeast (1). α-1, 3-Glucans can be extracted from cell walls with alkali; they precipitate when the alkaline extract is neutralized. Among the ascomycetous yeasts, only species of Schizosaccharomyces contain this polysaccharide. Among the yeasts with basidiomycetous affinity, Cryptococcus species have been reported to contain α-1, 3-glucan; but in species of Rhodotorula it is apparently absent (9).

THE NATURE OF ENDOGENOUS β-GLUCANASES IN YEAST

Because the microfibrillar glucan layer is insoluble in both alkali and acid, it seems reasonable to assume that the main event in removal, changes, or weakening of the cell wall must involve enzymatic hydrolysis of the long chains of β-1, 3-linked glucose residues in this polysaccharide. Although studies on the action of snail digestive fluid and microbial enzymes on yeast cell walls date back many years (1), the presence of endogenous β-glucanases in yeast itself was only implied on the basis of the fundamental changes occurring in yeast cell walls during vegetative and sexual reproduction.

Exo-β-glucanases

The first indirect evidence for the ability of yeasts to hydrolyze the β-1, 3-glucosidic linkage was obtained by Morris in 1955 (10) when he showed that certain yeasts were able to grow on extracts of Laminaria cloustonii fronds which contain up to 30% of laminarin on the dry weight basis. In 1963 Chesters and Bull (11, 12, 13) provided direct evidence for the excretion of extracellular laminarinase activity into the culture fluid of several fungi and of some unidentified marine yeasts grown on insoluble laminarin as the substrate. They were able to achieve a partial separation of both yeast and fungal laminarinase actvity into an exo-β-glucanase and an endo-β-1, 3-glucanase fraction. Presumably these enzymes were induced by the substrate on which the organisms were grown. Later, Brock (14) reported the partial purification of an intracellular β-glucanase from baker's yeast. His assays with whole cells as the enzyme

source revealed that unbroken yeast demonstrated negligible laminarinase activity, and no activity was detected in the growth medium of young cultures. Baker's yeast was, therefore, different in its physiology from the marine yeasts used by Chesters and Bull (11, 12). The purified enzyme from S. cerevisiae hydrolyzed both laminarin and the β-1, 6-linked polysaccharide pustulan with the production of glucose as the sole product, thus demonstrating that the intracellular laminarinase activity represented one or more exo-enzymes. p-Nitrophenyl β-D-glucoside (pNPG) was also hydrolysed. Because the ratios of the activities on the three substrates remained constant during enzyme purification and the three activities moved together during starch gel electrophoresis, Brock (14) postulated that a single enzyme was responsible for the three activites. An exo-β-glucanase with similar specificity was found in intracellular extracts of Hansenula wingei (15). He observed that laminarinase activity rose sharply during sexual cell conjugation in this yeast, indicating de novo synthesis of enzyme(s) involved in wall softening and cell fusion.

Abd-El-Al and Phaff (16) made a detailed study of the β-glucanases associated with S. cerevisiae, S. elegans, Kluyveromyces fragilis, and Hansenula anomala, including those secreted into the culture fluid by the last two species. Extensive purification of both intra- and extracellular laminarinases and comparison of their properties showed that each corresponding pair was similar and that all were exo-β-glucanases that hydrolyzed laminarin, pustulan as well as p-nitrophenyl β-D-glucoside. They provided extensive evidence that the actions on the different substrates were due to a single enzyme in each species of yeast. Because hypoiodite-oxidized laminarin (aldehydic end oxidized to a carboxyl group) was hydrolyzed but periodate oxidized laminarin (both ends modified) was not, it was concluded that the exo-β-glucanases hydrolyzed laminarin from the nonreducing end. All enzyme preparations had the same optimum pH for activity (5.5), but their kinetic constants on the various substrates differed markedly. Comparison of V_{max} and K_m values suggested that the rapid lysis of ascus walls in K. fragilis might be explained in terms of an enzyme with higher V_{max} and higher affinity to the ascus wall glucan than that present in baker's yeast. However, subsequent testing of purified exo-β-glucanases as described here on alkali-insoluble glucan or cell walls revealed only negligible activity.

Exo-β-glucanases with the general properties described above (sometimes referred to as unspecific or nonspecific exo-β-glucanases) have been shown in extracts of most yeasts studied so far. The species include Schizosaccharomyces versatilis and other fission yeasts (17), Candida utilis (18), Cryptococcus albidus var. aerius (19), Pichia polymorpha (20), Kluyveromyces aestuarii (21), K. phaseolosporus (22), and other Kluyveromyces species (23). Exo-β-glucanases from different yeasts vary in their kinetic parameters as shown in Table 1 for three substrates.

An immunological comparison of exo-β-glucanases from Kluyveromyces and Saccharomyces species by the microcomplement fixation technique (23) also showed fundamental differences. An exception was noted, however, for enzymes obtained from species shown to be synonymous by other molecular techniques. In these cases the immunological distances between the enzymes from such organisms were very small.

Table 1. Comparison of some physical-chemical properties of various yeast exo-β-glucanases

Species[a]	Laminaran		Pustulan		pNPG[b]	
	V_{max}[c]	K_m (mg/ml)	V_{max}	K_m (mg/ml)	V_{max}	K_m (mM)
Candida utilis (18)		1.2		0.7		3.1
Cryptococcus albidus var. aerius (19)		0.1		0.3		0.007
Hansenula anomala (16)	54	5	7.2	5.9	1.8	5.4
Kluyveromyces aestuarii (21)	260	1.7	3.9	4.5	24	5.0
K. fragilis (16)	83	1.2	7.4	1.8	2.2	0.6
K. phaseolosporus (22)		2		35.7		6
Saccharomyces cerevisiae (16)	9.5	14	3.8	3.6	0.7	215
Schizosaccharomyces japonicus var. versatilis (24)	350	6.2	52	166		

[a] The numbers after the species name are literature citations.

[b] pNPG, p-Nitrophenyl β-D-glucoside.

[c] V_{max}, Micromoles of glucose released per minute at 30°C and pH 5.5.

Atypical exo-β-glucanases. With improvements in the techniques for enzyme separation, a number of studies have revealed that many or possibly all yeasts synthesize additional exo-β-glucanases with substrate specificities different from the already discussed nonspecific exo-glucanases; i.e., the group of enzymes that hydrolyze laminarin, pustulan, p-nitrophenyl β-D-glucoside, but not periodate-oxidized laminarin. At least two and possibly more forms of atypical exo-β-glucanases have been recognized. In cell extracts of Pichia polymorpha and in culture filtrates of Candida utilis, an exo-β-glucanase was found that hydrolyzes only laminarin and p-NPG but not pustulan or periodate-oxidized laminarin (20, 25, 27). In contrast, cell extracts of Candida utilis contained only the typical, nonspecific exo-β-(1, 3) (1, 6)-glucanase (18). Kluyveromyces phaseolosporus (22) contains four enzymes with laminarinase activity. One of these was an exo-β-glucanase, found mainly in the cytoplasm, that hydrolyzed both laminarin and periodate-oxidized laminarin, suggesting the possibility that this enzyme can bypass the modified terminal molecule at the nonreducing end of laminarin. It also hydrolysed pustulan at a slow rate but not p-NPG. An enzyme secreted by protoplasts and intact cells of a strain of Saccharomyces cerevisiae (26) had similar properties

except that p-NPG appeared to by hydrolyzed. Recently, Villa and Phaff (unpublished) studied the β-glucanases in the effluent from a commercial baker's yeast plant. They also identified an exo-β-glucanase that could hydrolyse periodate-oxidized laminarin. Their enzyme, however, like that from K. phaseolosporus, did not hydrolyse p-NPG. In contrast to the typical, nonspecific exo-β-(1, 3) (1, 6)-glucanase which shows no activity on yeast cell walls or on alkali-insoluble yeast glucan, the exo-β-glucanase with activity on periodate-oxidized glucan does demonstrate lytic activity on isolated cell walls (22) and could play an important role in cell morphogenesis. A summary of the substrate specificities of the various exo-β-glucanases is given in Table 2.

Table 2. Substrate specificities of exo-β-glucanases

	Laminarin	Periodate-oxidized laminarin	Pustulan	p-NPG
Kluyveromyces phaseolosporus (22)[a]	+	+	+	-
Pichia polymorpha (20)	+	-	-	+
Candida utilis (culture fluid) (27)	+	-	-	+
Candida utilis (cell extract) (18)	+	-	+	+
S. cerevisiae (26)	+	+	+	+
S. cerevisiae commercial spent broth (unpublished)	+	+	+	-

[a]Numbers in parentheses denote references cited.

Endo-β-1, 3-glucanases

Although endo-β-1, 3-glucanases were initially not recognized while studies were being conducted on exo-β-glucanases in extracts or culture fluids of many species of yeast, Abd-El-Al and Phaff first observed endo-β-1, 3-glucanase activity in cell extracts and the culture medium of two apiculate (bipolarly budding) yeasts, Hanseniaspora valbyensis and H. uvarum (28). The partially purified enzyme had no activity on pustulan and hydrolyzed laminarin in a random pattern to oligosaccharides.

Barras (29) reported the presence of an endo-β-1, 3-glucanase in Schizosaccharomyces pombe homogenates. Most of the enzyme appeared to be associated with the cell walls. He noted that cell walls of this yeast underwent a slow autohydrolysis when incubated in a buffer solution. During this process, high-molecular weight, soluble glycans, and β-1, 3-linked oligosaccharides were liberated from the walls. At the same time a solubilization of the wall-associated β-1, 3-glucanase was observed, but because of instability of the enzyme attempts at recovery and further purification were not successful.

Work on endo-β-1, 3-glucanase associated with the fission yeasts was followed up by Fleet and Phaff (17, 30). They chose to work with Schizosaccharomyces versatilis, which proved to contain much higher levels of β-glucanase than S. pombe; and, in addition, the enzyme was much more stable. The cell extract and the culture fluid contained only non-specific exo-β-(1, 3) (1, 6)-glucanase (24); but the cell walls, when stored in buffer at pH 5.0, underwent a slow autohydrolysis with release of β-1, 3-linked oligosaccharides, glucose, and β-glucanase activity. During the autolytic process, the walls became very thin and up to 80% of the total wall-associated laminarinase activity could be recovered in soluble form. Of this activity, 87% proved to be exo-β-(1, 3) (1, 6)-glucanase (similar to that found in the cell extract and culture medium) and 13% was endo-β-1, 3-glucanase. The latter enzyme, when purified to electrophoretic homogeneity, was specific for the β-1, 3-glucosidic bond and hydrolysed laminarin randomly to laminaribiose and glucose. Its low K_m value (0.33 mg of laminarin/ml) indicates a much higher affinity for laminarin than shown by the various exo-glucanases (cf. Table 1). With laminarin, it was shown to have two cooperative active sites per enzyme molecule. The purified enzyme partially lysed Schizosaccharomyces walls, but complete lysis required the addition of α-1, 3-glucanase.

Preliminary evidence for the presence of endo-β-1, 3-glucanases in the cell walls of multilaterally budding yeasts was first supplied by Bacon et al. (31). Maddox and Hough (32) detected endo-β-1, 3-glucanase activity in autolysed brewer's yeast, and Arnold (33) partially purified such an enzyme from baker's yeast. Farkaš et al. (26) fractionated the β-glucanases excreted by protoplasts and intact cells of S. cerevisiae on DEAE-cellulose columns. One of the fractions, both from cells and protoplasts, was an endo-β-1, 3-glucanase. This enzyme was also present in the protoplast lysate. Other yeasts in which cell wall-associated endo-β-1, 3-glucanases were demonstrated (30) include Saccharomyces rosei, Kluyveromyces fragilis, Hansenula anomala, Pichia pastoris, and Candida utilis. Villa et al. (27) and Notario et al. (18) did not detect endo-glucanase activity in the culture fluid or in cell extracts of Candida utilis. This enzyme is, therefore, presumably tightly bound to the cell wall. In contrast, Villa et al. (22) demonstrated two endo-β-1, 3-glucanases in cell extracts and supernatants of lysed protoplasts of Kluyveromyces phaseolosporus. The enzymes, which were separated by a single passage of the crude extracts over Sephacryl S-200, differed in K_m value for laminarin, molecular weight, and pH-activity profiles but showed the same action pattern on laminarin with the production of laminaribiose and glucose as end products. Each of the endo-glucanases caused extensive lysis when baker's yeast cell walls were treated with them.

Less work has been published on the presence of lytic β-glucanases in yeasts with basidiomycetous affinity, even though β-glucans constitute one of the important cell wall components (1). Villanueva and Gacto (25) reported that β-1, 3-glucanase activity was lacking in cell extracts of Rhodotorula mucilaginosa, R. minuta, Sporobolomyces albidus, and S. salmonicolor but present in Cryptococcus diffluence. However, they did not determine the action pattern of the enzyme in the last species. Notario et al. (19), however, showed that the β-glucanase from Cryptococcus albidus var. aerius hydrolysed both laminarin and pustulan and is, therefore, not an endo-β-1, 3- but an exo-β-(1, 3) (1, 6)-glucanase. Meyer

and Phaff (9) demonstrated significant β-1, 3-glucanase activity associated with the cell walls of Rhodotorula glutinis, R. minuta, Cryptococcus terreus, and Cr. infirmo-miniatus, but β-1, 6-glucanase was absent. Since they did not determine the action pattern of the wall associated enzyme, it could be an endo-β-1, 3-glucanase, a specific exo-β-1, 3-glucanase, or a mixture of both. Further work in this area is needed to elucidate the role of β-glucanases in morphogenesis of basidiomycetous yeasts.

β-GLUCANASES AS GLYCOPROTEINS

Most investigators who purified β-glucanases from yeast did not determine if any of these enzymes contained carbohydrates in their molecules. Notario et al. (18), who isolated a nonspecific exo-β-(1, 3) (1, 6)-glucanase from cell extracts of Candida utilis and purified it to homogeneity (based on gel electrophoresis and ultracentrifugation), found that the enzyme contained 68% carbohydrate of which 53% was glucose, about 7% mannose and the remainder unidentified. A specific exo-β-1, 3-glucanase (without activity on pustulan) excreted into the culture fluid by C. utilis (27) contained 20% carbohydrate. Glucose was the principal sugar, but small quantities of mannose were also found. A nonspecific exo-β-(1, 3) (1, 6)-glucanase from cell extracts of Kluyveromyces aestuarii (21) was also shown to be a glycoprotein with 24% carbohydrate. In this case the component sugars were not determined. The two types of exo-β-glucanases found in Pichia polymorpha (20) also were shown to be glycoproteins with glucose as the principal sugar (34). Even though these exo-glucanases contained some mannose in their carbohydrate, they failed to interact with concanavalin A; and presumably the number of receptor sites in the carbohydrate moiety or its spacial configuration is inadequate for effective binding to the lectin. Probably not all exo-glucanases behave identically with respect to concanavalin A because Biely et al. (35) observed an incomplete precipitation of baker's yeast exo-β-glucanase in the absence of competing glycoprotein. Villa and Phaff (unpublished) also detected an atypical exo-β-glucanase in the spent culture fluid of a commercial yeast plant (Table 2) that could be adsorbed on concanavalin A-Sepharose 4B.

The glycosylation of endo-β-1, 3-glucanases appears to vary. The first enzyme to be investigated in this regard was from Schizosaccharomyces versatilis (30), and chemical analysis of the pure enzyme did not reveal any carbohydrate. It is possible that the fission yeasts are unique in this respect because subsequent investigators have shown by chemical analysis that several endo-β-1, 3-glucanases from multilaterally budding yeasts are glycoproteins with a strong affinity for concanavalin A. Biely et al. (35) showed that both intact cells and protoplasts of Saccharomyces cerevisiae excreted two exo-glucanases and one endo-glucanase into the culture fluid. The latter enzyme was quantitatively precipitated by concanavalin A, even in the presence of other mannoproteins. Villa and Phaff (unpublished) also isolated an endo-β-1, 3-glucanase from the spent broth of a commercial yeast factory by adsorption on concanavalin A-Sepharose 4B. Villa et al. (34) reinvestigated the β-glucanases associated with Pichia polymorpha (20) and tested the purified enzymes for carbohydrate content. As discussed above, the two exo-glucanases contained glucose as the main sugar; but the endo-β-1, 3-glucanase was shown to be a glycoprotein in which mannose was the predominant sugar (mannose:glucose ratio 1.7). The exo-glucanases of this yeast failed to

bind to concanavalin A, whereas the endo-enzyme was strongly bound. No details on the structure of the polysaccharides associated with the exo- and endo-enzymes are known at this time. Since both contain glucose and mannose (although in different ratios), structural details of the polysaccharides need to be elucidated to explain the differences in the binding to the lectin. Based on the knowledge that yeast mannan, a highly branched polysaccharide, binds to concanavalin A, it is likely that the polysaccharide associated with endo-β-1, 3-glucanases has a similar structure. The problem with studying the structural details of the carbohydrate moiety of such enzymes is, of course, to obtain a sufficient quantity of pure enzyme.

LOCALIZATION OF β-GLUCANASES IN YEAST

Because mechanically prepared cell extracts contained significant levels of laminarinase activity, it was originally thought that most of this activity was located in the cytosol and that in some species part of the enzyme is excreted into the culture medium during growth (16). Cytological studies by Moor (36) with S. cerevisiae demonstrated that the budding process is initiated by a striking, localized vesiculation of the endoplasmic reticulum. The vesicles appeared to fuse with the cell wall at the site of the prospective bud and were thought to contain cell wall modifying enzymes. Matile and co-workers (37, 38) subsequently demonstrated the association of laminarinase activity with vesicular type particles isolated from S. cerevisiae. Excess laminarinase or enzyme remaining after completion of the budding process could then diffuse through the cell wall and arrive in the culture medium.

More definitive results were obtained by comparing total laminarinase activity in mechanically prepared extracts of whole cells and lysed protoplasts of S. cerevisiae (37, 38). The protoplasts contained only 20% of the total laminarinase activity in whole cell extracts and part of this activity was sedimentable. It was, therefore, concluded that 85-90% of the laminarinase of whole cells is external to the plasmalemma. Farkaš et al. (26) confirmed the findings of Cortat et al. (38) and concluded that the β-glucanases of baker's yeast are located in the periplasmic space of the cell envelope. Evidence for such location in other species has been forthcoming. Villa et al. (22) found that protoplasts of Kluyveromyces phaseolosporus contained only 5% of the laminarinase activity of whole cell extracts. Villanueva and Gacto (25) were able to inactivate selectively nearly all laminarinase activity of Candida utilis by mild mineral acid treatment without affecting significantly the viability of the cells or an internal marker enzyme β-glucosidase. The same was shown for the laminarinases of Pichia polymorpha (20). Since during cell breakage the glucanases of yeasts appear to a large extent in the soluble extract, these enzymes do not appear to be covalently linked to the cell wall polysaccharides. Mechanical cell disruption must cause extensive damage to the wall-adhering membrane causing release of the enzyme. It appears, at least in some yeasts, that the endo-β-1, 3-glucanase is more tightly bound to the wall than the exo-enzymes (16, 30).

FUNCTION OF YEAST ASSOCIATED β-GLUCANASES

At least five functions for yeast cell wall-associated β-glucanases can be lised. (i) The ability of some yeasts to utilize laminarin or its oligosaccharides for growth (10, 11) endows such species with a nutritional

advantage by being able to grow in a marine environment on decomposing seaweeds or in soil at the expense of autolysing fungi (e.g., mushrooms). The exo-β-glucanases discussed in the previous sections can supply the cell with glucose for growth.

(ii) Directly preceding and during early stages of bud initiation the β-glucanase level of synchronously dividing cells rises (32, 38) and the specific activity per unit dry weight of cells decreases during the period of bud maturation. The rise in enzyme level coincides with the secretion of glucanase-containing vesicles in the area where a bud is to emerge (38). When the secreted glucanases reach the cell wall glucan, the endo-β-1, 3-glucanase component is thought to cause breaks in the existing glucan network in a random pattern. Chain length adjustment can then be accomplished by the exo-β-glucanases present. During this cell wall softening process, expansion of the bud becomes possible; but this must be supplemented by the concerted action of UDPG glucosyltransferase(s) which insert new glucosyl units into the expanding wall (39). In the presence of 2-deoxyglucose, which interferes with the synthesis of UDPG, insertion of new glucosyl units in the areas of glucan synthesis (such as the apical portion of young buds) is blocked and lysis of the cell takes place (39). Nonbudding cells contain glucanases in the periplasmic space; but the enzymes do not hydrolyse the cell wall glucan or cause lysis, either in the presence or absence of 2-deoxyglucose. A clear explanation for this lack of activity is not available; but presumably in the mature cell these enzymes and their substrate become physically isolated from each other, thus preventing hydrolysis.

(iii) The β-glucanase complex also plays an important role in the lysis of asci of those species of yeast that release their spores upon maturity (e.g., species of Kluyveromyces, Pichia, and Hansenula). Such yeasts generally contain higher β-glucanase levels than those that do not liberate the spores from the ascus (16, 38). In Kluyveromyces phaseolosporus (22) two endo-glucanases and an exo-glucanase (with activity on periodate-oxidized laminarin) were shown to have lytic activity on isolated cell walls, and these enzymes are thought to be responsible for the lysis of ascus walls. There is no informatin on the factors which allow these enzymes to initiate the lytic process when the cell wall becomes an ascus wall. It may be that even in mature cells there is a balance between synthetic repair processes and lytic activity of the glucanases in such yeasts; but that after the formation of the ascospores, the glucan-synthesizing enzymes are locked away in the spores, leaving the glucanases free to lyse the ascus wall.

(iv) Brock (40) reported that cell fusion during sexual conjugation in Hansenula wingei required protein synthesis, and he postulated that this protein represented cell wall-softening glucanases acting on the mating cells. Evidence for this was provided later when he showed that the β-1, 3-glucanase level increased sharply during cell conjugation (15). A similar phenomenon was shown to occur in conjugating cells of Schizosaccharomyces versatilis cells (17). It is possible that the mating pheromones produced by haploid, heterothallic cells and which cause copulatory outgrowth in cells of complementary mating types are directly or indirectly responsible for the induction of glucanases involved in cell wall softening.

(v) Yeast autolysis. As a final example of the role of β-glucanases in yeast, their participation in autolytic processes may be mentioned (32, 33). Although cytoplasmic protein degradation constitutes the main event in autolysis, limited hydrolysis of cell wall polysaccharides also takes place. The difficulty of complete auto-digestion of isolated Schizosaccharomyces walls was pointed out by Fleet and Phaff (30); and these authors obtained preliminary evidence that laminari-saccharides may inhibit the activity of endo-β-1, 3-glucanases. Nevertheless, ascus walls of Schizosaccharomyces lyse completely after the spores are mature; and the different susceptibilities of cell walls and ascus walls to autolysis cannot be explained at this time. It may be postulated that during the process of ascosporulation a highly lytic β-glucanase is synthesized in the cytoplasm and that such an enzyme initiates the lysis. Much remains to be learned on the regulation and physiology of the β-glucanase complex in yeast.

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ENDOGENOUS LYTIC ENZYMES AND THE YEAST CELL WALL

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INTRODUCTION

Studies carried out in the last few years have brought important changes in the concept of the biological role of the yeast cell wall. Today the cell walls are not envisaged as inert structures with mere mechanical functions but as surface organelles which show specific turnover and support various catalytic processes related to cell economy and morphogenesis. Shape and, in most instances, even the cell viability are dependent upon the integrity of the cell wall. In spite of the importance of the yeast cell wall, knowledge on the molecular basis of its organization and differentiation is far from complete. As pointed out in a recent review (1), probably one of the factors responsible for this situation is the lack of a united approach from the several areas engaged in explaining at the molecular level the biogenesis and morphogenetic changes of this complex structure.

Among the multidiscipalinary treatments that have focused on the yeast cell walls, the study of specific lytic enzymes able to act exogenously on the cell wall has been important in trying to develop an integrated view of its architectural organization (2-4). More recently, interest has been increasing on the occurrence of endogenous enzymes capable of modifying the main structural polymers of the cell wall and to account for the various changes that take place in the wall during the life cycle of yeast cells. In this contribution we will briefly summarize some selected aspects of the cell wall of yeasts to illustrate what we know and what still remains to be known about this structure and the related enzymes presumably involved in its modifications.

STRUCTURAL ELEMENTS OF THE YEAST CELL WALL

The general design of the cell walls is similar even at very different biological levels. They all contain structural polymers which are responsible for their mechanical strength and also for the cell morphology, forming the framework upon which soluble polymers are found. In the various biological groups that possess

cell walls, different types of molecular solutions to the problem of conferring mechanical strength on the wall are found. The framework in *Saccharomyces cerevisiae* is a group of microfibrillar polymers consisting of glucose units joined mainly by β (1:3) linkages. Early evidence that this component is the main structural polymer in *S. cerevisiae* walls was obtained when it was found that after extraction of soluble wall components by alkaline and acid treatment the residue showing the call shape was the β-glucan (5). The β-glucans are embedded into a matrix mainly formed by glycoprotein complexes generically called yeast mannan. These glycoproteins are formed by mannose units linked to polypeptide backbones. The protein moiety of these molecules may act as a bridge between several mannan subunits giving rise to a polymer with a very high molecular weight which also contains phosphate (6). The mannose units of mannan are not β-linked as in higher plants but attached through α-glycosidic bonds. Another structural polymer also present in the yeast cell wall, although generally in much smaller quantities, is chitin, a polysaccharide formed by repeating units of N-acetylglucosamine. In budding yeasts this compound is mainly located at the neck between mother and daughter cells originating the primary septum and supporting one of the most conspicuous formations on the cell wall : the bud scar (7,8).

The above components, glucan, mannan-protein complexes and chitin, account for the bulk of the dry weight of the yeast cell wall. Some other minor components, such as lipids, have also been described but their contribution to the architecture of the wall is unclear. In addition to providing an envelope responsible for both the compressional and tensile strenght of the cell, the wall also defines a periplasmic space above the plasma membrane in which several exoenzymes have been located. Many of the cell wall associated enzymes are in turn glycoproteins with mannan as part of their molecule (9). We will describe some of the prominent characteristics of the major components just mentioned and afterwards discuss the role of endogenous lytic enzymes relevant to the morphogenesis of the yeast cell wall.

YEAST GLUCAN

Glucan is the main component of *S. cerevisiae* cell walls, representing as many as 60 % of the wall. Chemical analysis of the yeast glucan has given conflicting results but it has been recently shown that, as usually prepared, it is heterogeneous and contains both β(1:3) glucan components. Baker's yeast cell walls contain at least three kinds of glucan. An alkali-soluble form has been characterized that previously was discarded during extraction and purification of the mannan fraction. This glucan represents about 20 % of the cell wall and behaves as a homogeneous molecular species in ultracentrifuging, gel filtration and electrophoresis (10). Structural analysis of this fraction revealed that most of the linkages are of the β(1:3) type, although approximately 10 % of β(1:6) linkages are also present. Examination by electron microscopy of the cell walls after

alkaline extraction of this glucan reveals that an amorphous surface layer also containing the mannan has been removed but the cells exhibit the original shape, suggesting therefore, that this glucan species does not significantly contribute to the architectural features of the wall. This glucan contains a small proportion of mannose and protein, indicating that in the cell wall it may form part of a glucan-mannan protein as suggested previously (11).

The alkali-insoluble glucan comprises in turn two components. The major one represents about 85 % of the crude alkali-insoluble fraction and it is a branched β(1:3) glucan containing about 3 % of β(1:6) linkages (12). The minor component is somewhat anchored to the major one, from which it can be isolated by extraction with hot acetic acid. It consists of a highly branched β(1:6) glucan with a small proportion of β(1:3) interchain linkages (13). When the alkali-insoluble glucan is rendered soluble by various enzymic or chemical treatments the original shape of the cell virtually disappears leaving the bud scars as residues of the original wall. Some results indicate that the major glucan with predominant β(1:3) linkages form an inner layer covered by a shall of the predominantly β(1:6) linked polysaccharide (5,14). There is also evidence that the alkali-soluble glucan is located between the alkali-insoluble glucan and an outer layer of a mannan-protein complex thus acting as a bonding material (10).

The physical state of the structural glucan in native intact cell walls has been surrounded with some controversy centred upon whether or not fibrills of glucan exist as such in the cell wall. The answer has been in dispute for years. Parital hydrolysis by boiling in dilute hydrochloric acid makes the residual alkali-insoluble glucan assume a crystalline arrangement termed hydroglucan that is characterized by a microfibrillar appearance. Some considerations suggested that the microfibrillar disposition of this fraction in the cell wall was an artifact (5). Hydroglucan probably represents a linear polymer of β(1:3) linked glucose residues arising by elimination of branch points what might enable this component to aggregate, butthe configuration of the intact structural glucan is more complicated. However, there are studies in which microfibrills have also been observed under other circumstances. The existence of a fibrillar component has been recently demonstrated by methods as mild as enzymic treatment by bacterial glucanases (14). Densely interwoven microfibrills were also demonstrated in the same study in untreated cell walls by electron microscopy after negative staining. It would thus appear that the glucan might present a crystalline-like configuration at least partially but that this structure is normally difficult to visualize among the amorphous wall material. Nevertheless, the physical basis for this conformation is unclear. A fibrillar network is also frequently observed during the process of cell wall regeneration by yeast protoplasts, but this may not correspond to glucan (15).

Because of the structural function of glucans it would be important for the understanding of the cell wall to know how they

are synthesized and laid down. Unfortunately, the data are sparse. Cell-free extracts capable of synthesizing glucan have been recently described (16, 17). Some results point to the fact that UDP-glucose might be the nucleotide involved in glucan synthesis (16, 17) and glucose analogs have been found to interfere with glucan synthesis and to cause cell lysis (18). At present knowledge on the synthesis of these components in yeasts can rely only on indirect evidence of glucan incorporation into the wall. It was considered that synthesis of both glucan and mannan occurs continuously along the whole cell cycle (19). However, a reevaluation of the data suggests that the rate of synthesis is not constant but considerably reduced at the time of cell division and in the prebudding phase (20).

It should be finally noted that glucan molecules in which the glucose units are mostly α(1:3) linked occur in other yeasts such as *Cryptococcus* and *Schizosaccharomyces* species (21, 22). In others, such as *Rhodotorula* and *Sporobolomyces*, glucan polymer responsible for the structural functions carried out by glucan in most yeast cell walls (23-25).

ENDOGENOUS YEAST GLUCANASES

Attention has recently focused on the role of endogenous lytic enzymes able to modify the structure of the yeast cell wall through the morphogenetic processes of the yeast life cycle. Cell conjugation, sporulation, budding and cell wall extension during vegetative growth as well as yeast autolysis are phenomena in which the yeast cell wall appears to be subjected to partial lysis or softening by controlled cell-associated enzymes. Since glucan is considered the main structural component of the cell wall the participation of β-glucanases in these processes has been frequently claimed. Earlier interpretations of cell wall morphogenesis implicated both sulfhydryl cell wall content and the enzyme protein disulfide reductase in the weakening of the wall structure (26). However, the activity able to cleave disulfide crosslinks among cell wall proteins is not present in all yeasts (27).

Brock reported the existence of an intracellular β(1:3) glucanase in baker's yeast capable of hydrolyzing both laminarin and pustulan as well as p-nitrophenylglucoside (28). Glucose was the only product liberated when acting on the corresponding glucans. The enzyme activity exhibited by this enzyme on the three substrates appears to be the common pattern of substrate specificity to most yeast exo-splitting β-glucanases. A similar enzyme was later reported in *Hansenula wingei* and since the activity sharply increased during conjugation it was postulated that the activity could be involved in cell wall softening during the mating of the cells (29). Phaff's co-workers also examined several yeast species for the presence of this activity (30). In all cases exoglucanase activity was detected in cell extracts and the extracellular activity, when present, had identical properties to the intracellular one. As noted above, the enzymes showed unspecific activity on laminarin, pustulan

and PNPG. The enzyme from *S. lactis* also cleaved both β(1:3) and β(1:6) glucan in an exosplitting fashion as well as the synthetic low-molecular weight glucoside (31). However, the exo-β-glucanase from *Candida utilis* appears to be specific for laminarin only; it had no activity on pustulan or gentibiose although it was able to hydrolyze PNPG (32). An enzyme with the same anomalous substrate specificity has also been reported in *Pichia polymorpha* (33).

TABLE 1 Characteristics of purified β-glucanases from *Cr. albidus* (38).

Properties	G-I	G-II	G-III	G-IV
Elution from DEAE-Sephadex at pH 7.0 (NaCl)	0.25 M	0.5 M	0.6 M	not retained
Optimum pH for activity	5.0	5.5	5.0	5.0
% Activity after 10 mins. at 50°C	41	23	16	16
Isoelectric point	4.1	4.1	3.9	6.0
Molecular weight ($x10^3$)	20	10	21	21
Action on laminarin	endo	exo	exo	exo

In contrast to the enzymes just described the β(1:3) glucanases from other yeasts hydrolized laminarin to oligosaccharides and did not cleave postulan nor PNPG (34). Endo-β(1:3) glucanase activity was also described as occurring in the fission yeast *Sch. pombe*(35). The enzyme was mostly associated with the cell wall and solubilization resulted in increased instability. A detailed study of the activity in *Sch. versatilis* revealed the occurrence of two glucanase enzymes; a soluble exoglucanase activity accounted for most of the total activity and a minor endoglucanase was also found to be bound to the cell wall (36). The activity of the isolated exoglucanase on alkali-insoluble glucan was only negligible while the endoglucanase caused extensive cell wall lysis. This may serve as an indication that the former enzyme does not play a major role in cell wall lysis. Evidence for the occurrence of endoβ(1:3)glucanase activity has been additionally shown in a great number of yeast species which show multilateral budding (33,37). In many cases the occurrence of both exo- and endo-splitting enzymes has later been described and the reinvestigation of the enzyme composition in various species previously studied has revealed that the glucanase system may be more complex than originally thought. For instance, up to at least four different glucanase enzymes can be distinguished in *Cr. albidus* on the basis of physico-chemical properties and substrate specificity (Table 1).

Interest in the significance of these enzymes was further promted by Johnson's observations on the cell wall lysis induced by glucose analogs (18). A generalized model was suggested by which changes in the glucan layer of the wall would occur by a concerted participation of both endogenous glucanases and glucan synthases; the first enzymes would create new points of insertion by cleaving some glucan linkages on which the synthase would act by adding new sugar units. The net balance of both activities would then determine either autolysis or extension of glucan during growth. A similar suggestion about glucanase involvement in cell wall changes has been also supplied by examining cell growth and cell wall integrity in several sprofitic yeast species which grow normally at 27°C but lyse when shifted to 37°C in the absence of osmotic stabilization (39). UDP-glucose, a precursor for glucan biosynthesis, accumulates into the medium at the restrictive temperature and a fissure develops where normally a bud would appear. No differences were noted in exoglucanase activity of intact cells, cell homogenates or filtrates of cultures grown at the two temperatures. Unfortunately the levels of endoglucanase, which is the enzyme more strongly suspected to be involved in yeast cell wall biogenesis, were not measured in these studies. The above observations could be interpreted to mean that aberrant wall formation and lysis in unstabilized medium at 37°C is the result of an inbalance between the effective utilization of the new wall synthesis precursor at the site of bud formation and the action of repair enzymes. This model is quite suggestive but, although glucanases have been located at the level of the cell wall (32), practically nothing is known about the actual location of the glucan synthases enzymes in yeasts (16, 17).

Additional circumstantial evidence has accumulated to indicate a role for glucanase enzymes in the various processes of cell wall reorganization that accompany yeast morphogenesis but no direct proof has yet been established (40). A screening about endogenous glucanase enzymes in several yeast species revealed an initial correlation between presence or absence of the corresponding structural glucan on the wall and the presence or absence of enzyme activity (32). A sudden increase in enzyme activity was also noted during the transition to arthrospore forms in dimorphic Endomycetales, where the mycelial phase has a lower glucan content as compared to yeast-like cells, and based on these observations there were suggestions about glucanase implication in cell fragmentation. However, alternative explanations for this and other results are possible. Fungal glucanases appear to be constitutive enzymes that may be regulated by catabolite repression (41) so that glucose depletion could be the cause of both a parallel increase in glucanase activity and the simultaneous triggering of other unrelated phenomena.

Studies with synchronous cultures of *S. cerevisiae* gave support to a functional role of yeast glucanases in cell budding (42). In those studies an increase in the enzyme activity at the phase of growth closely preceding the initiation of budding was observed. However, the significance of these findings is not clear and might be the subject of controversy. The involvement

of these enzymes in the budding process during the course of the cell cycle in *S. cerevisiae* has been extensively investigated in this laboratory by zonal fractionation of cell cultures and the results are presented in an accompanying contribution to this volume (43). Based on earlier reports on the possible role of these enzymes in conjugation (29, 44), recent studies have also clarified that the mating process is not necessarily accompanied by sharp increases in glucanase activity. A detailed examination of the exo- and endoglucanase activity at various times during the process revealed that no change evident in the two activities although a glucanase e after mating and zygote formation had (43). It seems therefore that if gluca way implicated in cell wall precopulatory tion should be mediated by changes in the the activity or cell wall substrate accessibility rather than by changes in enzyme levels.

Despite the extensiveness of data regarding yeast β(1:3)glucanases, direct experimental evidence is lacking to confirm unequivocally the hypothesis about their role. The most rational approach towards the elucidation of the functionality of these enzymes should be the isolation and characterization of mutant cells altered in their production. The apparent difficulty in trying to obtain such kind of viable mutants was previosly emphasized as an argument to further supporting the idea of their involvement in the normal cell development (31). In this context, the isolation of a mutant from *S. cerevisiae* defective in the production of β(1:3)exoglucanase has recently been obtained in this laboratory (45). The selection procedure after mutagenesis was based on the capacity of the periplasmic exoglucanase to cleave synthetic glucosides.

The isolated mutant retained the β(1:3) endoglucanase activity but the exo-splitting enzyme was totally undetectable in cell extracts (Fig.)1 . Moreover, a protein band identified as the enzyme in electrophoretic runs of extracellular products secreted by the wild type strain could not be detected by analyzing the proteins secreted by the mutant. The altered character was stable and showed Mendelian segregation; since the endoglucanase activity was not modified by the mutation it is clear that the two activities behave as the products of different, unrelated nuclear genes. The mutation does not seem to affect the physiology of the yeast cells since no significant alterations were observed when different parameters of the mutant and the wild type strains were comparatively analyzed (46). Thus, these results strongly argue against a critical involvement of this enzyme activity in yeast cell wall morphogenesis although a major role in those processes could be exerted by the endoglucanase enzyme, an important part of which is also located in the periplasmic space (46). In support of this proposal the authors pay attention to the fact that the endohydrolytic enzyme shows a much higher affinity for yeast β(1:3)glucan than the exohydrolytic enzyme. Obviously there is no doubt that the obtention of non-lethal mutants deficient in β(1:3) endoglucanase would de-

finitively clarify the controversial role of β-glucanases in modifications of the yeast cell wall.

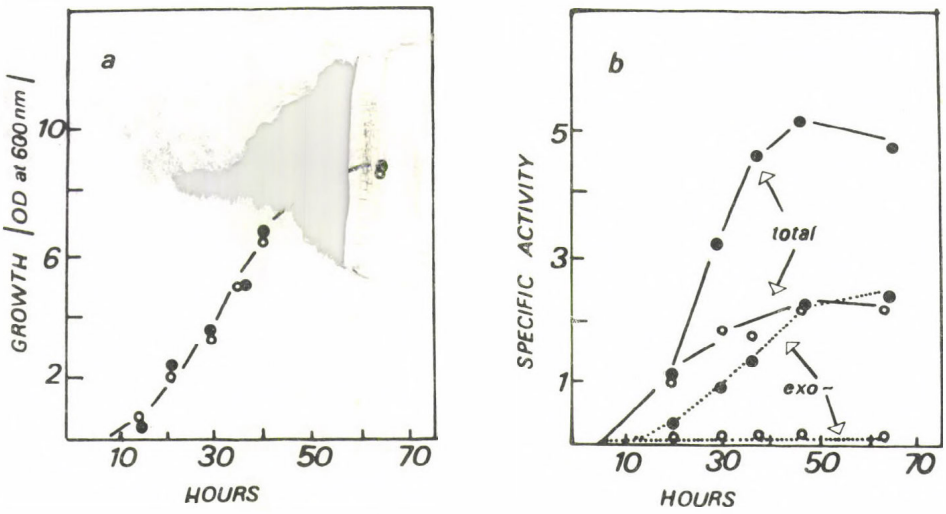

Fig. 1 Growth (a) and production of β-glucanases (b) in *S. cerevisiae* S288C (●) and C31C (o). (Modified from (45)).

CHITIN AND YEAST CHITINASES

As already mentioned, chitin is another structural polymer of the yeast cell wall. Independent pioneer work showed that chitin is mainly concentrated as a disk-like plug at the bud scar region in *S. cerevisiae* (7,8). Based on its rather restricted spatial location and on the fact that its synthesis takes place only during a limited portion of the cell cycle a model has been proposed to account for the localized distribution of chitin in space and time (47); several assumptions of the model have been proved although recent data point to the existence of alternative activation mechanisms for the zymogen chitin synthase (48, 49). New structural approaches on chitin biosynthesis have also emerged from the discovery of vesicles that act as conveyors for chitin synthase from the endoplasmic reticulum to ist final destination on the plasma membrane (50). A recent reexamination of chitin location on the cell wall by using a specific lectin as cytochemist marker has recently indicated that it is not exclusively limited to the bud scar area but is also present all around the cell wall (51). However, quantification of this material is missing. Deposition of this polymer on the zone of the wall newly growing during mating has also been

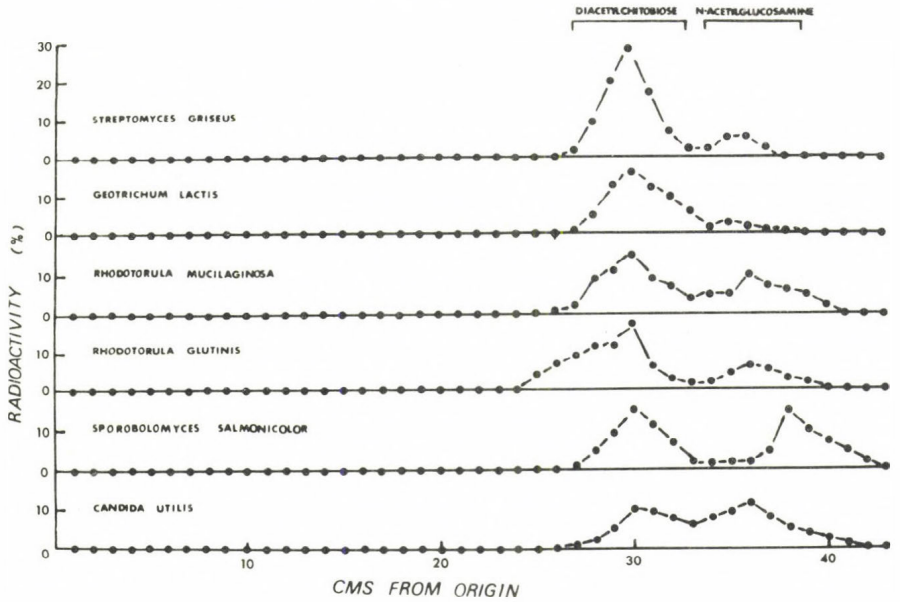

Fig. 2 Chromatographic profiles of the hydrolisis products of chitin by cell extracts from different yeasts and by chitinase from *Str. griseus*.

described (52).

While the synthesis and location of chitin in budding yeast has been mostly studied in *S. cerevisiae* there are however other species in which larger amounts of this component are present (23-25). In those cases chitin might follow a different arrangement and play an important structural role in the cell walls. In this context it is of interest to report that we have recently initiated a study on the presence of chitinases in yeasts. By using radioactive substrate obtained by acetylation of chitosan (53, 54) we have been able to demonstrate the occurrence of such enzyme activity in cell extracts from several species. Yeast chitinases show a hydrolytic pattern similar to that reported for the actinomycete *Streptomyces griseus* releasing mostly diacetylchitobiose from chitin in species in which N-acetylglucosaminidase activity could not be detected (Fig. 2). Contrariwise to other lytic enzymes from yeasts the activity was not evident extracellularly nor was it significantly inactivated under conditions in which periplasmic enzyme activities are lost (32). The enzyme from the dimorphic species *Geotrichum lactis* has been further investigated. In the micelial phase about 30 % of the activity is found associated to membranes while almost 70 % of the activity represents soluble intracellular enzyme; an opposite distribution is found in yeast-like unicellular forms. By centrifugation of cell extracts from protoplasts in density gradients the particulate

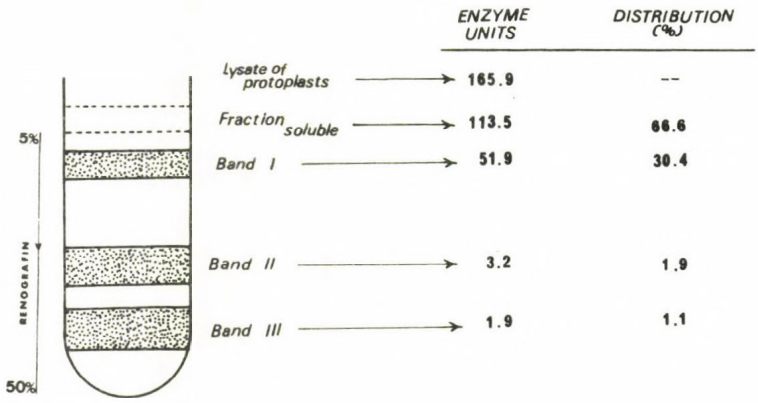

Fig. 3 Percentage distribution of chitinase activity from protoplast lysates of the mycelial form of *G. lactis* in Renografin gradients.

enzyme has been preferentially located in a fraction of low density membranes that might correspond to vacuoles (Fig. 3). The significance of these findings in morphogenesis remain unknown.

OTHER CELL WALL RELATED LYTIC ENZYMES FROM YEASTS

Yeast mannan is considered as a cementing polymer without skeletal properties although it is well established that the action of chelating and thiol agents on the protein moiety of these complexes allows cell wall softening (55) . As far as we know there is a single reference to the presence of mannanase activity in yeasts (56) and some preliminary data from our laboratory also confirm the presence of such activity in autolysates from *S. cerevisiae*. Since mannan is at the surface of the wall it is a candidate with a role to play in cell surface morphogenetic phenomena (51). Changes in this component at the early steps of conjugation have been detected but the results suggest that the modifications can be explained by an altered rate in the synthesis of the wall polymer rather than by involvement of hydrolytic activities (57).

The presence of xylomannans among basidiomycetous yeasts is known although the relevance of these polymers in the architecture of the cell walls has not been evaluated (22, 38). The occurrence of a xylanase activity has been reported (58); the activity assayed in permeabilized cells is periplasmic endohydrolytic in nature and highly unstable (Fig. 4). Nevertheless, the significance of this type of activity in cell wall related events remains to the established.

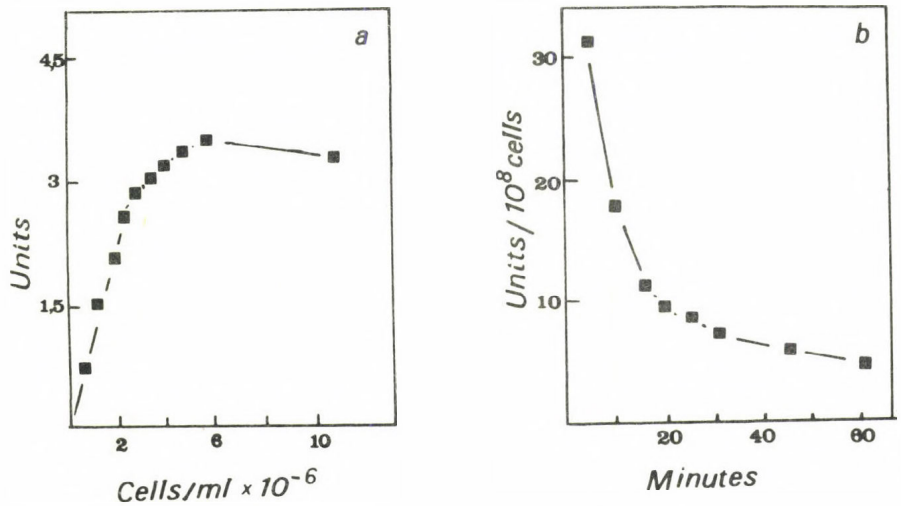

Fig. 4 Dose dependence (a) and time course inactivation at 37°C (b) of xylanase activity in *Cr. albidus*. (Modified from (38)).

The presence of α(1:3) glucan has also been demonstrated in cell walls of various yeasts and its occurrence is suspected in others (21, 22). This component may be more widely distributed in the cell wall of yeasts than assumed previously and α(1:3) glucanases could be present among the enzymes produced by microorganisms capable of lysing these walls. As an example it should be mentioned that *Sch. pombe* cells fail to produce protoplasts when treated with glusulase unless the enzyme complex is supplemented with α(1:3) glucanase of microbial origin (unpublished results). This enzyme activity has also been implicated in morphogenesis of filamentous fungi (59). Following the assumption that α(1:3) glucanases should occur in yeasts that presumably contain this particular wall polymer as a structural entity efforts have been made to detect the presence of these enzymes in yeast cells. The results from a survey for the activity revealed the absence of enzyme activity in several species of the genus *Schizosaccharomyces* while assays in species of *Cryptococcus*, *Rhodotorula* and *Endomyces* gave indication for the presence of a rather labile α(1:3) glucanase which was mostly associated with cell wall fractions (60). Interestingly, the existence of the substrate polymer

has not been reported in *Rhodotorula* and, conversely, no activity was detected in genera in which cell wall α(1:3) glucan has been demonstrated. These results yield the idea that the presence or absence of the enzyme activity cannot be used as a marker to unequivocally postulate the corresponding presence of the substrate at the level of the cell wall. In addition, the rapid inactivation of the enzymes so far investigated suggests that it is probable that the quantitative estimations of the activities detected are not representative of their actual or potential activity "in vivo" and that the apparent absence of the enzyme in yeasts known to contain the polymer may deserve more careful examination.

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CHARACTERIZATION OF HYDROLASES IN PROTOPLASTS, MYCELIUM, SPORES AND ABERRANT TUBES OF *TRICHODERMA VIRIDE*

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SUMMARY

Hydrolases of the filamentous fungus *Trichoderma viride* mainly those involved in morphogenetic processes, have been studied in spores, mycelium, protoplasts of spores and mycelium and aberrant tubes produced by regenerating protoplasts whose cell wall composition, lacking chitin, differs from that of the normal mycelium (1).

There is an increase in β-(1-3), β-(1-6) and α-(1-3) glucanase during germination. At this time the appearance of chitinase, absent in spores, takes place. Protease and cellulase also increase though less significantly. Some of these enzymatic activities show a new increase in the autolytic phase at the end of the growth curve. The main hydrolases located in aberrant tubes are the same as those detected in mycelium. It is noteworthy that these aberrant tubes have chitinase in spite of the lack of chitin in their walls. Studies of these enzymes in protoplasts indicate their location in the periplasmic space.

The results lead us to the conclusion that β-(1-3) and β-(1-6) glucanase and chitinase have a morphogenetic role while α-(1-3) glucanase and cellulase can be considered mostly as metabolic enzymes.

INTRODUCTION

Our understanding of the structure and biological function of a certain number of polysaccharides located in the cell wall of the fungi has been considerably improved through the study of the hydrolases involved in the synthesis and degradation of these polymers. In fungi most hydrolases have been described as constitutive (2) and attributed a fundamental role in morphogenesis since they hydrolize the polysaccharides during the processes of germination and of growth and elongation of the cell wall (3,4). There are also numerous fungi which excrete into the

culture medium considerable amounts of hydrolases when they are growing in the presence of cell wall polysaccharides (5,6).

Numerous polysaccharases which degrade the polymers of the cell wall of several fungi can be induced in the filamentous fungus *T. viride*. Since α-(1-3) glucanase, β-(1-3) glucanase, chitinase and cellulase have been described (7,8,9) and because of the antagonistic role in soils of *T. viride* this fungus can be regarded as an important agent in the control of several fungal diseases.

Although the study of hydrolases in *T. viride* has been quite exhaustive there have been very few reports concerning their involvement in morphogenesis. In our research, the main hydrolases of *T. viride* have been characterized with regard to their morphogenetic role. Our results confirm the close relationship between hydrolases and the regulation of cell wall biosynthesis.

MATERIALS AND METHODS

Strain and growth conditions

The strain of *T. viride* used belongs to the Spanish Type Culture Collection (CECT 2423), Salamanca. The microorganism was growing in a GAE medium (glucose, asparagine and yeast extract) (10) in flasks inoculated with a spore suspension 10^6 cell/ml) and incubated at 28°C with shaking. The inoculum was obtained from culture grown on solid GAE for 48 h. Samples were withdrawn at different times, centrifuged at 2000x g for 10 min and then washed with distilled water. Finally they were kept at -20°C for further analytical assays.

Protoplasts were obtained by a previously described procedure (1) and the regeneration process carried out in the Winge medium (glucose 2% w/v); yeast extract 0.3% w/v) plus 0.7 M $MgSO_4$, with shaking. After 10-12 h nearly all the protoplasts had produced a large and irregular tube (11). Such aberrant tubes were collected by centrifugation at 30.000 x g for 40 min, washed twice with 0.7 M of $MgSO_4$ and kept at -20°C.

Analytical methods

In some experiments, 0.1 ml of a mixture toluene-ethanol (1:1 vol/vol) was added to each sample. In most cases, samples of spores, mycelium and aberrant tubes were disintegrated by shaking with powdered glass (Ballotini no. 12) in a Braun MSK system for 1 min.

The measurement of the activities was made by taking 0.5 ml of the appropriate substrate - that is, 1% laminarine (Koch-Light) to determine β-(1-3) glucanase activity; 1% pseudonigeran (5) was used to assay α-(1-3) glucanase activity; 5% chitin (Sigma) for chitinase; 1% caseine (Sigma) for protease; 0.5% pustulan (12) for β-(1-6) glucanase and 1% carboxymethylcellulose (Sigma) for cellulase activity. The substrates were buffered with sodium acetate buffer (0.05 M, pH 5.5), except for the protease assay in which Tris-HCl buffer (0.1 M, pH 8) was used. The samples were incubated at 30°C for 30 min. The reaction was stopped

by adding 2 ml of $ZnSO_4$ (5%) and 2 ml of 0.1 M $Ba(OH)_2$. The samples were then centrifuged at 2000 x g for 10 min. Glucanase and protease activities were determined by taking 1 ml samples from each supernatant and measuring the products of hydrolysis, i.e. reducing sugars amino acids, respectively.

Protoplasts were collected by centrifugation at 10.000 x g for 30 min, washed twice with 0.7 M $MgSO_4$ and resuspended in distilled water. 0.5 ml of the lysate was incibated with 0.5 ml of the appropriate substrate and the products of hydrolysis measured as described above.

Total reducing sugars were determined as glucose-equivalents (13). Amino acids were measured by the method cited in (1).

Proteins were extracted with 1 M NaOH for 1 h at room temperature and measured by the method described in (14) using bovine albumin as a standard. The values given for hydrolase activities are expressed as specific activity; that is, µg of substrate liberated per min and per mg of protein.

RESULTS AND DISCUSSION

Tables 1 and 2 show the different values obtained for hydrolase activities in spores and mycelium of *T. viride*. There is an increase of β-(1-3) and β-(1-6) glucanase and protease during the germination process, whereas an increase of chitinase takes place later, during the phase of active growth (10-24 h).

TABLE 1. Enzyme activities detected in toluenized cells of *T. viride*

Activities	Dormant spores	Turgent spores	Mycelium 18 h old	Mycelium 24 h old
β-(1-3) glucanase	6	30	33	46
β-(1-6) glucanase	7	15	21	26
α-(1-3) glucanase	0	0.5	1	1
Cellulase	0	0	0.8	1
Protease	0	1	2	3
Chitinase	0	0	0.15	0.5

This is the case for all the above-mentioned enzymes listed in tables 1 and 2. However, in table 1, α-(1-3) glucanase seems to increase slightly but this increase is not reflected in table 2. This is also the case for values obtained for cellulase.

In case of dormant spores, enzyme activity measurements are generally lower than those of cell-free extracts. This is probably due to the fact that those substrates of high molecular weight cannot pass through the membrane and are, therefore, only partially hydrolysed. This could be the case for protease and cellulase whose substrates, caseine and cellulose, have a high molecular weight.

TABLE 2. Enzyme activities detected in homogenized samples (cell-free extracts) of *T. viride*

Activities	Dormant spores	Turgent spores	Mycelium 18 h old	Mycelium 24 h old
β-(1-3) glucanase	20	25	30	48
β-(1-6) glucanase	18	20	17	24
α-(1-3) glucanase	0.8	0.5	1	0.5
Cellulase	0.6	1.2	1.5	1
Protease	0.6	1.2	1	2
Chitinase	0	0	0.5	5

The morphogenetic function of an enzyme is based mainly on two factors: its relation with the cell wall polymers (4) and its fluctuations throughout the cell cycle (15). The polymers located in the cell wall of the mycelium of *T. viride* are mainly β-(1-3) and β-(1-6) glucan and chitin. The spore cell wall lacks chitin and, as can be seen in tables 1 and 2, spores also lack chitinase, which suggests a morphogenetic role for β-(1-3) and β-(1-6) glucanase and chitinase. This suggestion is also supported by the change of concentration of these activities throughout the growth curve. A more exhaustive study carried out on β-(1-3) and β-(1-6) glucanases shows that their concentration in the cell increased considerably after 3-4 days of culture, when the autolytic processes took place. β-(1-3) glucanase underwent an increase of 100 fold after 4 days of culture, supporting the theory that there is a very close relationship between the increase in β-(1-3) glucanase activity and autolysis (15). β-(1-6) glucanase shows a big increase of activity between 8 to 12 h of culture as if it were mainly involved in the germination process but the increase at the end of the growth curve is not as large as that of β-(1-3) glucanase (data not shown).

TABLE 3. Enzyme activities detected in aberrant tubes of *T. viride*

β-(1-3) glucanase	7
β-(1-6) glucanase	10
α-(1-3) glucanase	3
Cellulase	4
Protease	18
Chitinase	1

The data shown in table 3 refer to toluenized aberrant tubes. The hydrolases determined in these structures are the same as those of normal mycelium, though their concentration is quite different; a higher value for β-(1-6) glucanase and a much higher one for protease can be observed. The most surprising data were those referring to chitinase since aberrant tubes lack chitin. The hydrolase results obtained from toluenized aberrant tubes show detectable levels for protease and cellulase. These activities were located in cell free extracts of dormant spores but not in toluenized samples. This might be due to the structure of aberrant tubes, which is less compact and therefore the toluene can work more effectively on the membrane, letting bigger molecules, like caseine or cellulose, pass through it. The

data obtained from enzymatic and chemical analysis (1) show that the composition of the aberrant tubes is different from that of normal mycelium; the use of fluorescent brighteners (16) also seems to show that the synthesis of new material and its incorporation in the cell wall is different. Our results showing such differences in concentrations of hydrolases between normal mycelium and aberrant tubes support the previous conclusions.

TABLE 4. Enzyme activities detected in protoplasts of *T. viride*

Activities	Spore protoplasts	Mycelium protoplasts
β-(1-3) glucanase	6.3	9.0
β-(1-6) glucanase	10.0	6.5
α-(1-3) glucanase	0.15	0.3
Cellulase	0	0
Protease	1.3	0.6
Chitinase	0	0.5

Table 4 shows that most of the enzymes characterized in cell-free extracts can also be detected in lyzed protoplasts, though in a lower concentration. This seems to indicate that all the hydrolases are located either between the membrane and the cell wall or somehow associated with the cell wall, as already suggested by previous experiments (4, 17). Possibly the activities detected in protoplasts are due to enzymes recently synthesized or which are being transported to the periplasmic space. The variations in enzyme concentration between protoplasts and cell-free extracts oscillate between 1/2 and 1/5. This supports the extracellular character of the location of these particular enzymes in *T.viride*.

In two experiments, several fractions were separated by centrifugation at different speeds, after the cells had been disintegrated. The determination of β-(1-6) glucanase shows some activity in all the fractions but a big and unique peak was detected in the fraction associated with the cell walls. This result supports the suggestion that the enzyme is somehow associated with the cell wall "in vivo" (data not shown).

In most cases the results obtained by comparing hydrolases activities between protoplasts of spores and those of mycelium are similar to those obtained when turgent spores and mycelium are compared, which would support the morphogenetic role attributed to β-(1-3) and β-(1-6) glucanases and chitinase.

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REGULATION OF FUNGAL 1,3-β-GLUCANASES

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INTRODUCTION

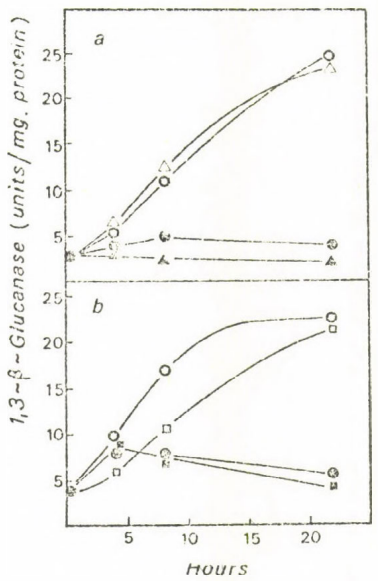
In the last few years our interest has focused on the glucan degradative enzymes produced by several species which have 1,3-β-glucan as one of the major components of their cell wall. The importance of these enzymes relates mainly to the possible role that they might play in fundamental processes for cell morphogenesis, such as growth and extension of the cell wall (1), bud emergence, daughter cell separation and mating (2-4).

Central to the problem of interpreting the role of glucanases is the study of their regulation. Although the production of these enzymes is very well documented, data on their regulation are very scarce and some of them concern 1,3-β-glucanases of bacterial origin, which probably serve a function very different from that of many of the fungal 1,3-β-glucanases. One of the few examples of glucan degradative enzymes on which some data about its regulation have been reported is the exo--1,3-β-glucanase of the Basidiomycete QM806 (5). A control, at the level of synthesis, seems to operate since the production of this enzyme is repressed by glucose and only carbon source deprived cells synthesize it in very high amounts. But glucose also seems to trigger the specific inactivation of the enzyme when added to derepressed cells indicating that degradative controls are also operative (5). On the other hand, the exo--1,3-B-glucanase of Saccharomyces cerevisiae has been reported to be synthesized, once per mitotic cycle, paralleling the time of bud emergence (6) and an induction of 1,3-β-glucanase has been shown to take place, in Hansenula wingei, upon mixing two strains of opposite mating types (4).

This paper presents an account of the studies that we have carried out in the last few years, in order to gain understanding of the regulation of 1,3-β-glucanase synthesis in fungal cells. The wide diversity which exists among these enzymes must be taken into consideration. A single species can produce more than one 1,3-β-glucanase whose mode of action could be either endo-hydrolytic or exo-hydrolytic or even a combination of both. Three species of filamentous fungi and a yeast have been used for our investigations.

CATABOLITE REPRESSION OF 1,3-β-GLUCANASE

In some species, the production of 1,3-β-glucanase is catabolite repressed. For example, the filamentous fungus Penicillium italicum (Fig. 1) produces a very low amount of 1,3-β--glucanase when incubated in a synthetic medium with an excess of glucose or other easily metabolizable sugars, such as fructose or sucrose, which support active growth. On the other hand, when the medium contained, either a very limited amount of glucose or a high concentration of other sugars such as lactose or galactose, which are not readily assimilated, the production of 1,3-β-glucanase was significantly activated leading to a very high increase in the level of specific activity. Very similar observations were made in Neurospora crassa indicating that 1,3-β-glucanase production is also catabolite repressed in this fungus (8).



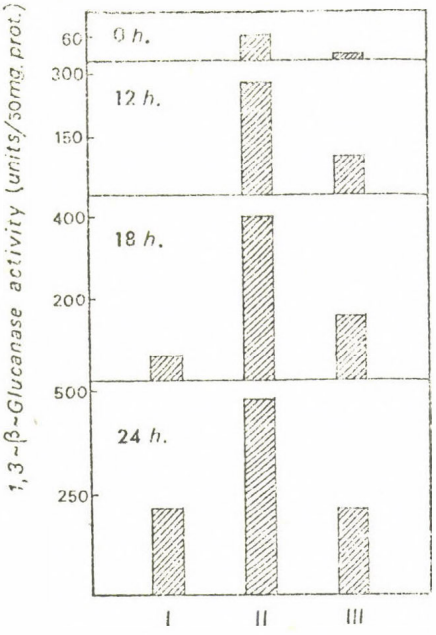


Fig. 1.(left). Effect of different sugars on the production of 1,3-β-glucanase by Penicillium italicum. Actively growing cells were transferred to synthetic media containing a sugar as the only carbon source. Sugar concentrations were as follows: 0.2% glucose (o), 3% glucose(●), 3% sucrose (■), 3% fructose (▲), 3% galactose (□), 3% lactose (△). Values indicate specific activity of 1,3-β-glucanase in cell extracts. Data taken from ref. 7.

Fig.2.(right). Relative proportions of 1,3-β-glucanases I,II and III in Penicillium italicum after different periods of incubation in a medium low in glucose content(10mM).

Extracts of cells were prepared and analyzed by ion-exchange column chromatography. Data taken from ref. 10.

A more detailed examination of the P. italicum 1,3-β-glucanase system has been carried out. With the use of RNA and protein synthesis inhibitors we have shown that the glucose effect must be exerted at a pretranslational level which could either be m-RNA synthesis or some stage of the process involved in its maturation or stabilization (9). Three 1,3-β-glucanases, which have been named 1,3-β-glucanase I, II and III, are produced by P. italicum and the evolution of their relative proportions, when the mycelium was incubated in a low-glucose medium, is indicated in Fig. 2. Relevant to the possible function of these enzymes could be the following facts. Enzymes II and III are the only ones produced during active growth and derepression leads to much higher levels of them. These two 1,3-β-glucanases can bind to the cell walls and hydrolize their components in vitro. Derepression of 1,3-β-glucanases is also paralleled by the in vivo mobilization of a significant amount of cell wall glucan. On the other hand, 1,3-β-glucanase I cannot be detected in actively growing cells, appears only under conditions of glucose deprivation and neither binds to the cell walls nor is capable of hydrolyzing them in vitro (11).

According to these results 1,3-β-glucanases II and III must be the enzymes involved in the modification of cell wall glucan during active growth, if that event is needed for morphogenesis and cell wall extension (1). They should also be responsible for the mobilization of cell wall glucan under derepression conditions, which could take place either with the purpose of supplying energy or be coupled to a morphogenetic event such as conidiation. A similar situation exists in Aspergillus nidulans, where the degradation of cell wall α-1,3-glucan takes place in glucose starved mycelium and supplies energy needed for cleistothecium formation (12). On the other hand, the induction of 1,3-β-glucanase I must be directed towards the hydrolysis of external glucan with metabolic purposes.

GLUCOSE INSENSITIVE SYNTHESIS OF 1,3-β-GLUCANASES

The glucose repression type of control does not operate in all fungal species. For example, in Trichoderma viride and Saccharomyces cerevisiae the production of 1,3-β-glucanase takes place during active growth in the presence of glucose, so that increasing levels of specific activity were observed when the cells were incubated in synthetic media in the presence of an excess of this sugar. Moreover, resting cells, deprived of glucose, either failed to produce the enzyme or produced it in much smaller amounts (Fig. 3). Therefore, a constitutive synthesis of 1,3-β-glucanase takes place in Trichoderma viride and S. cerevisiae suggesting that glucan degradative capacity is required for the active growth of the cells.

The described two different modes of regulation of 1,3--β-glucanases synthesis indicate that the diversity in the properties of 1,3-β-glucanases extends to their carbon source

regulation and complicates the problem of interpreting their

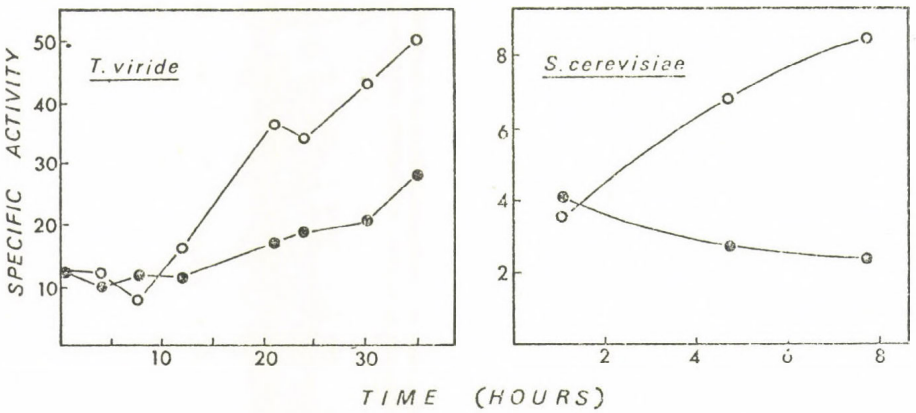


Fig.3. Effect of glucose on the production of 1,3-B-glucanase by two fungal species. Logarithmic phase cells were transferred to synthetic media with either a high concentration of glucose (180 mM) (o) or a low concentration of this sugar (5 mM) (•). The specific activity of 1,3-β-glucanase was assayed in cell extracts with laminarin as a substrate. Data taken from ref.8.

role in the fungal cell. In our studies on S. cerevisiae we have called the attention to the fact that the low pHs, which sometimes are attained during growth could cause an inactivation of 1,3-β-glucanases because they are periplasmically located enzymes. In these cases, unless the medium is properly buffered, the measurement of the levels of these enzymes could be totally meaningless (8). This inactivation probably can account for the low glucanase content which is frequently observed in stationary phase yeast cells.

1,3-β-GLUCANASES IN THE Saccharomyces cerevisiae LIFE CYCLE

Vegetative growth is not the only phase of the S.cerevisiae life cycle in which the participation of 1,3-β-glucanases might be needed. In order to gain insight on the regulation of these enzymes we have examined the evolution of the levels of glucan degradative activities during the mitotic cycle, and the mating and sporulation processes. Two 1,3-β--glucanases are produced by S.cerevisiae during vegetative growth (13, 14). One is an exo-splitting enzyme, active on laminarin (1,3-β-glucan) and on the synthetic derivative p-nitrophenyl-β-D-glucoside (p-NPG). The other is an endo-glucanase which hydrolyzes laminarin and periodate oxidized laminarin (1,3-β-glucan with the end sugar residues modified) but not on pustulan or p-NPG.

In order to study the synthesis of both enzymes, during the mitotic cycle, asynchronous cultures were fractionated

and the glucanases content of the different fractions, which contained populations of cells corresponding to the different stages of the mitotic cycle, was examined. As shown in Fig.4a, the levels of both enzymes doubled in the fractions corresponding to the transition from the S to the G_2 phase. These resuls clearly indicate that the synthesis of exo- and endo-1,3-β-glucanase, during the mitotic cycle, takes place in a stepwise manner, once per cycle upon the transition from the S to the G_2 phase. This contrasts with the report of Cortat et al. (6) which suggest that the synthesis of exo-1,3-β-glucanase, in S. cerevisiae, parallels budding and therefore must occur during the G_1 phase. On this basis these authors postulate the involvement of the exo-1,3-β-glucanase in the budding process. The reason for this discrepancy is not apparent to us, but in any case the conclusions attributing a certain role to an enzyme, on the basis of its moment of synthesis in the mitotic cycle, should be regarded with caution. One can think that enough exo-1,3-β-glucanase can be synthesized, at the G_2 phase, even if it is needed at a later stage. Moreover, we have shown that a S. cerevisiae mutant, defective in exo-1,3--β-glucanase, grows and buds normally (14).

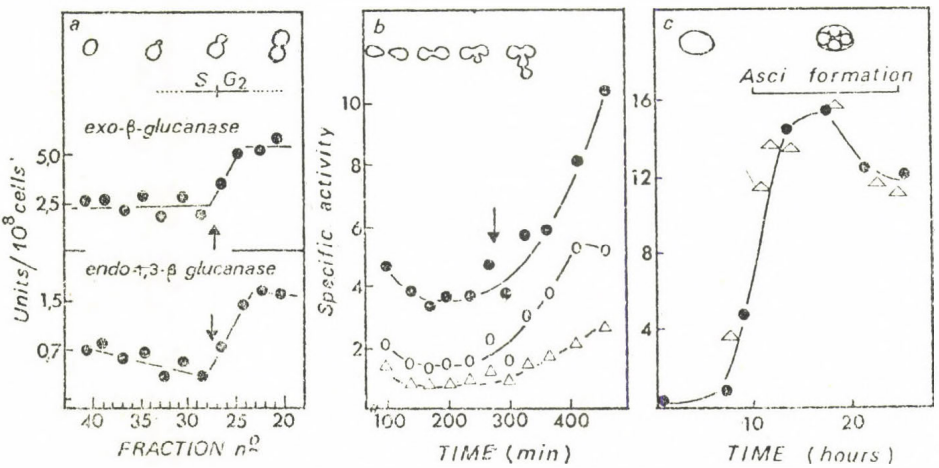


Fig.4. Levels of 1,3-β-glucanases in Saccharomyces cerevisiae during the mitotic cycle (a) and the mating (b) and sporulation processes (c). Values represent: a) Exo- and endo-1,3-β-glucanase content, per 10^8 cells, in synchronous fractions obtained by fractionation of an asynchronous culture. b) Specific activity against laminarin (•) oxidized laminarin (o) and pustulan (△) when a and α strains were mated, c) Specific activity against laminarin (△) and oxidized laminarin (•) in cells of the diploid strain AP-1 incubated in sporulation medium.Drawings illustrate the different periods of the S. cerevisiae life cycle. Data taken from ref. 15.

Fig. 4b shows the evolution of the levels of glucan degradative enzymes during the mating process. When populations of cells of a and α mating types are mixed together, growth is arrested at the G_1 phase due to the hormonal stimulus of the opposite mating type (16), while zygote formation occurs. During this period, the specific activity of cell extracts against laminarin and oxidized laminarin remained constant. However, when zygote formation was completed and growth of zygotes and non-mated haploid cells resumed, the specific activity against those substrates started to increase very significantly. Therefore, mating in S.cerevisiae does not seem to lead to an activation of 1,3-β-glucanases. If these enzymes are involved in the process of cell fusion, the amounts of them, present in the cells before mating, must be enough to accomplish this role. It is only growth resumption that restores the production of 1,3-β-glucanases. The situation seems to be different in other yeast, Hansenula wingei, since Brock (4) has reported an induction of 1,3-β-glucanase when strains of this species mate in a nitrogen-free medium, incapable of supporting growth.

The sporulation process involves very important changes from the morphological and biochemical points of view. Our studies with a highly sporulating strain, S.cerevisiae AP-1, indicate that this is accompanied by significant changes in the levels of 1,3-β-glucanases (Fig. 4c). The specific activities against oxidized laminarin and laminarin, very low at the time of transfer to the sporulation medium, increased significantly and reached a maximum after 18 hours, paralleling the apearance of asci. Contrary to what happens in vegetative cells, where the activity against laminarin is much higher than against oxidized laminarin, in sporulating cells the specific activities reached values which were very similar and much higher than we ever observed during vegetative growth.

This kind of evidence is consistent with the idea that the sporulation process, in S.cerevisiae, is accompanied by specific changes which affect the 1,3-β-glucanase complement of the sporulating strain. It is known that during sporulation many enzymes, which do not seem to be specifically involved in this process, are, nevertheless, synthesized in an ordered sequence which very closely reproduces the timing of the mitotic cycle (17). However, the induction of glucan degradative activities during sporulation takes place in a manner which is very different from that of the mitotic cycle and therefore it seems to be specifically triggered by the sporulation event. An alteration in the relative proportions of the two enzymes produced during active growth and/or the induction of a new 1,3-β-glucanase might account for the observed changes. One of the few examples of enzymes, shown to be activated by the sporulation events in S. cerevisiae, are glycogen hydrolyzing activities (18).

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THE STEREOLOGY OF PROTOPLASTS FROM *Aspergillus nidulans*

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SUMMARY

The ultrastructural heterogeneity of protoplasts released from the hyphae of *Aspergillus nidulans* during staged lytic digestions was investigated using stereological methods. Protoplasts released after 1h, with either KCl or $MgSO_4$, as osmotic stabiliser, were small and had dense granular cytoplasm but were characterised by the presence of many vesicles. As the lytic digestion proceeded emerging protoplasts were larger with less dense cytoplasm and fewer vesicles. The high frequency of vesicles in the 1h fraction is consistent with the hypothesis that these protoplasts originate from the vesicle-rich apical zones of the hyphae.

INTRODUCTION

During protoplast isolation the highly integrated structure of the fungal hypha becomes sub-divided into discrete heterogeneous units (1). Variation in protoplast morphology was first recognised by Bartnicki-Garcia and Lippman (2) in *Phytophthora cinomomii*. The heterogeneity of *Aspergillus nidulans* protoplasts was first reported by Peberdy and Gibson (3) and later confirmed in ultrastructure observations (4).It was suggested that protoplasts released during the first hour of lytic digestion originated from apical regions of hyphae and those appearing later came from more distal regions,progressively further from the hyphal apex. Electron micrographs of 'early' and 'late' protoplasts showed ultrastructural differences that could be released to their original hyphal location.

An ultrastructural study was undertaken, in order to investigate and quantify the extent of protoplast heterogeneity occurring during the lytic digestion of *A. nidulans* mycelium, in different stabiliser solutions which induce different patterns of protoplast release (5,6). Stereological

measuring procedures were applied to thin sections to provide a quantitative analysis of cytoplasmic components present in each protoplast sample.

MATERIALS AND METHODS

Organisms:-

Aspergillus nidulans, BDUN 33, (University of Nottingham collection) was maintained on malt extract agar. Mycelium was grown in mineral salts medium (7) supplemented with glucose ($10g.l^{-1}$).

Trichoderma harzianum, CBS 354-33, was maintained on TLE medium, supplemented with ball-milled chitin and laminaria meal and solidifed with agar (5).

Protoplast isolation:-

Protoplasts were prepared by the method of Peberdy and Isaac (5) using KCl and $MgSO_4$ as stabilisers. Protoplasts were harvested from these mixtures at 1,2 and 3h after the start of digestion, residual mycelium being replaced in the lytic mixture after each harvest. Preparations were harvested and washed free from lytic enzyme as previously described (6).

Preparation of material for electron microscopy:-

Material was prepared by the method of Gibson and Peberdy (4). Thin sections were cut with glass knives, on a Porter-Blum ultramicrotome. Sections were expanded using trichloroethylene, mounted on formvar coated grids, stained with lead citrate (8) and thoroughly washed under a stream of running water. Specimens were examined using an AEI Corinth 275 electron microscope.

Preparation of material for stereological analysis:-

The primary sample consisted of three blocks of embedded protoplasts from each treatment. One silver section was mounted from each block, and from each section 13 micrographs were recorded at random (using a systematic procedure) at 15,000 x magnification. Usually a single protoplast profile almost filled the field at this magnification.

Micrographs, recorded on 70mm film, were transferred to 35mm film and analysed using a back-projection and counting system similar to that described by Weibel, Kistler and Scherle (9).

Stereological procedures:-

A Weibel and Knight (10) multipurpose test system was used, having a test area of 6 x 6.06 Z (Z = 0.5μm). The dimension Z was chosen as being the square root of the mean mitochondrial profile area in a small smaple of pictures.

For volumetric analysis the protoplasts were sub-divided into the

following compartments: ground cytoplasm, mitochondria, endoplasmic reticulum, lipid inclusions, vesicles, nuclei and vacuoles.

Although some of the compartments being measured were undergoing major volume changes during the experiment, the major components under study (ground cytoplasm, mitochondria and vesicles) were consistently found in all micrographs of 1h incubated material and so it was decided to use single pictures as representative samples. A total of 39 such samples was measured for each treatment; sample parameters were calculated and the final results represent the means of these. Data are expressed on the basis of volume fraction per unit volume of total protoplast or of cytoplasm (protoplast less nuclei and vacuoles).

RESULTS

Figure 1 shows the major morphological characteristics of protoplasts liberated from A. nidulans mycelium. The average size of protoplasts increased as mycelial digestion proceeded.

Table 1:-

Volumetric composition of protoplasts per unit volume of whole protoplast (%)

		VOLUME FRACTION OF WHOLE PROTOPLAST (%)						
STABILISTER	TIME	C	M	V	N	E	I	Vs
KCl	1h	57.90 ± 2.34	13.66 ± 1.25	4.51 ±1.08	4.65 ±1.07	1.18 ±1.77	1.11 ±1.09	16.47 ± 2.03
	2h	66.71 ± 1.44	12.70 ± 1.00	4.40 ±0.86	8.43 ±1.02	1.60 ±0.76	3.32 ±0.90	4.06 ± 0.75
	3h	68.92 ± 1.49	9.06 ± 0.98	9.48 ±1.50	9.12 ±1.37	0.45 ±0.17	1.57 ±0.04	1.49 ± 0.03
$MgSO_4$	1h	64.30 ± 1.56	16.10 ± 1.09	4.34 ±1.04	4.34 ±0.83	- -	0.73 ±0.21	10.39 ± 1.25
	2h	61.90 ± 1.65	12.00 ± 0.75	13.30 ±1.59	8.29 ±0.98	1.34 ±0.68	1.89 ±0.41	1.49 ± 0.45
	3h	62.10 ± 1.96	7.47 ± 0.68	18.30 ±2.51	9,40 ±1.66	3.58 ±0.17	1.50 ±0.36	0.99 ± 0.36

C - cytoplasm; M - mitochondria; V - vacuoles; N - nuclei, E - endoplasmic reticulum; I - lipid inclusions; Vs - vesicles.

Table 1 shows the volumetric density of components, as a percentage composition of unit protoplast volume. In both stabiliser systems, a high volumetric density of michondria per unit volume was seen in early protoplasts. A similar pattern was seen with regard to vesicle contents. Early protoplasts had a very significantly higher volumetric density (10-16%) than later fractions (1-2%) per unit volume of protoplast. The large

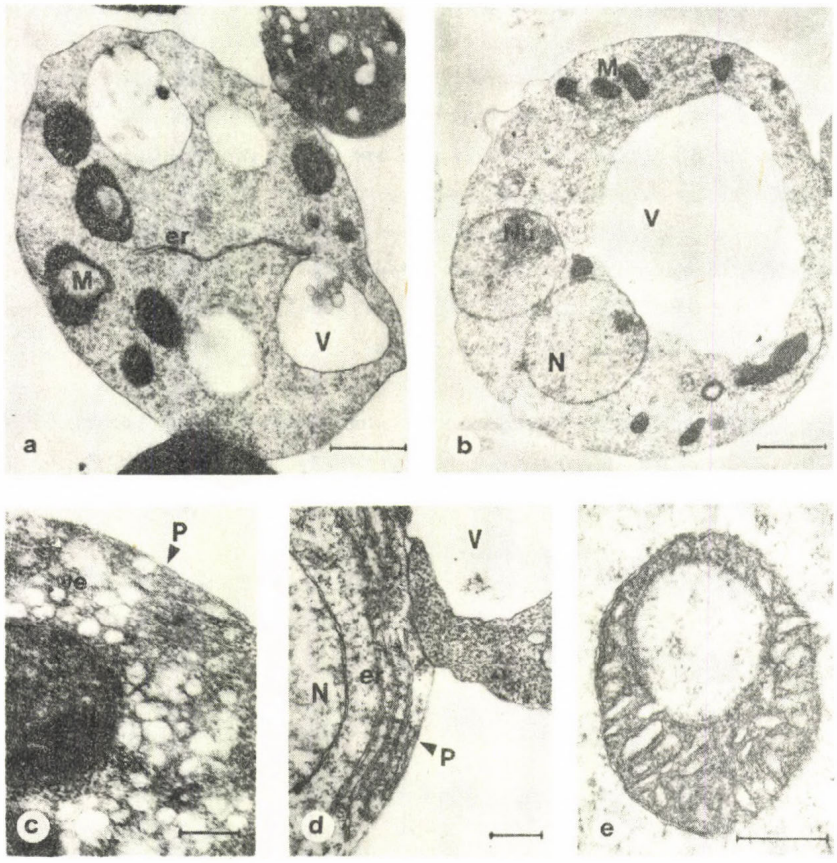

Figure 1:- a) Protoplast from 3h incubation in KCl stabiliser. Bar marker represents 0.05μm. b) Protoplast from 3h incubation in $MgSO_4$ stabiliser. Bar marker represents 0.05μm. c) Section of protoplast from 1h incubation in $MgSO_4$ stabiliser to show vesicles. Bar marker represents 0.25μm. d) Section of protoplast from 3h incubation in KCl stabiliser to show endoplasmic reticulum. Bar marker represents 0.25μm. e) Section of mitochondrion from a 3h $MgSO_4$ stabilised protoplast. Bar marker represents 0.25μm.

M - mitochondria, N - nuclei, Nu - nucleolus, V - vacuole, P - plasmalemma, er - endoplasmic reticulum, ve - vesicles.

volume occupied by vesicles was particularly significant in early KCl-stabilised protoplasts.

Considering the volume fractions of these two organelle types in relation to the unit volume of cytoplasm, a different picture emerges (Table 2). The mitochondrial unit volume remained relatively constant in samples whereas the volume fraction of vesicle material is significantly higher in the 1h fractions produced in both KCl and $MgSO_4$.

Table 2:-

Volume fractions of vesicles and mitochondria per unit volume of cytoplasm (%)

TIME	VOLUME FRACTION OF CYTOPLASM (%)			
	KCl Treatment		$MgSO_4$ Treatment	
	Vesicles	Mitochondria	Vesicles	Mitochondria
1h	17.86 ± 1.95	14.78 ± 1.24	10.82 ± 1.26	17.47 ± 1.24
2h	4.32 ± 0.77	14.75 ± 1.14	1.47 ± 0.53	15.67 ± 1.14
3h	1.69 ± 0.39	10.86 ± 1.15	1.23 ± 0.45	10.16 ± 0.81

The volumetric density of vacuoles showed a reversed pattern; these occupied a larger volume in protoplasts produced later in digestion. The increase in this volume was observed mainly between 2-3h in KCl-stabiliser whereas in $MgSO_4$-systems a more significant increase was seen from 1-2h (4.5-13.5%), a smaller increase occurring from 2-3h (13.5-18%). The volume occupied by nuclei was also higher in later fractions.

DISCUSSION

From the results obtained it was seen that sequentially produced protoplasts differed significantly in ultrastructural characteristics. This was seen with both the osmotic stabilser systems used.

Vesicular and vacuolar contents, together with the general cytoplasmic organisation around these organelles, were the main factors by which protoplasts could be identified. The high vesicular content of hyphal apices has been well discussed in a range of taxonomic groups (11, 12, 13). From the numerous studies undertaken, concerning apical growth mechanisms, Bartnicki-Garcia (14) proposed a model implicating apical vesicles in tip growth, which is now generally accepted. The high vesicular contents of early protoplasts suggests particularly that these

may have their origin at tip regions. In addition the high ribosomal density in the cytoplasm of this fraction was characteristic. The vesicular content of early protoplasts (1h) from $MgSO_4$-stabilised preparations was lower than that from KCl-stabilised preparations. Early $MgSO_4$-stabilised protoplasts were larger and may therefore have included a higher proportion of sub-apical cytoplasm than corresponding protoplasts from a KCl-system. Thus early KCl-produced protoplasts (1h) may be composed of more specifically "apical" cytoplasm, which may account for their higher vesicle content.

In addition the vacuolar content of the later formed protoplasts suggests that these may be released from older hyphal regions. Vacuoles are found in distal zones of hyphae (13, 12) as has been shown in A. nidulans mycelium (16). $MgSO_4$-stabilised protoplasts have a higher vacuolar volumetric density than KCl-stabilised protoplasts. This may be directly attributable to the different osmotic effects of the stabiliser concerned, but it has been shown that early protoplasts allowed to remain in the lytic mixture for up to 3h (17), did not acquire the characteristics displayed by later formed protoplasts.

Protoplasts lend themselves well to stereological analysis since any one electron micrograph may contain one whole protoplast profile. The magnification used was sufficiently low for each micrograph to yield a representative section area and high enough to allow identification of component organelles. The random sampling techniques employed here yielded a representative sample of protoplast material but it must be stressed that the data collected were estimates from which a mean value, covering the whole sample, was obtained, on which statistical analysis was performed. For any material there will be systematic errors of measurement inherent in the system (18). This study involved the comparison of various samples of similar nature and corrections for some factors were unnecessary since each sample was similarly affected (18). Special reference must be made to membrane structures which may become difficult to recognise at some sectioning angles due to the Holmes effect (19,20). Cisternal spaces may be difficult to recognise. The data for endoplasmic reticulum quoted here is uncorrected for any such error.

ACKNOWLEDGEMENTS

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THE CELL-WALL ENZYMOLYSIS TESTS : TAXONOMIC STUDY OF GENERA *CANDIDA* AND *TORULOPSIS*

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ABSTRACT

The lytic action of purified enzymes on the cell-wall of yeasts led to the elaboration of two tests named cell-wall enzymolysis tests. The enzymes released sphaeroplasts of the treated yeasts. A discrimination between Ascomycetes and Basidiomycetes was obtained by using a reducing agent associated with 1,3-β-glucanase (cell-wall enzymolysis test 1 or C.E.T. 1). A separation between two subclasses of Basidiomycetes was obtained by using 1,4-α-glucanase associated with 1,3-β-glucanase (C.E.T. 2). The use of these two cell-wall enzymolysis tests was very useful to study Fungi Imperfecti yeasts. In this work, the two tests were applied to two genera : *Candida* and *Torulopsis* whose taxonomy is presently a subject of debate. They are separated into Ascomycetous and Basidiomycetous yeasts, and a key of identification is proposed.

INTRODUCTION

In previous works (1, 2, 3, 4), we have tried to prove that release of sphaeroplasts from yeasts using a definite enzymatic system was a very useful means of separating Ascomycetes from Basidiomycetes. The standardization of such a methodology led us to carry out the cell-wall enzymolysis tests, or C.E.T. The use of purified 1,3-β-glucanase (extracted from QM 806 Basidiomycete culture) associated with a reducing agent was valuable for separating the two classes (Ascomycetes and Basidiomycetes). We named it C.E.T. 1. The use of purified glucanases (1,3-β-glucanase and 1,4-α-glucanase) extracted from *Trichoderma viride* culture was valuable for separating the Basidiomycetous yeasts into two subclasses. This C.E.T. 2 was very useful to compare for instance *Cryptococcus* (first sub-class) and *Rhodotorula* (second sub-class) (6, 7). These two C.E.T. were applied to two genera of Fungi Imperfecti yeasts which are very close : *Candida* and *Torulopsis*.

MATERIAL AND METHODS.

The strains of *Candida* and *Torulopsis* were collection strains ; most came from the Centraalbureau Voor Schimmelcultures (C.B.S.), Baarn, Netherlands, and the others from the Institut Pasteur, Paris (I.P.) as well as from our

own collection (I.V.P.). The strains were cultivated on a synthetic medium (Yeast Morphology Agar, Difco B.393). Following three successive 48 h subculturings at 26°C, all strains were grown for 15 h just prior to the enzymic treatment.

The 1,3-β-glucanase was extracted from Basidiomycete QM 806 culture. After precipitation of proteins, the enzymic extract was filtered through a Sephadex G 100 gel column (3, 4). The enzymes of *Trichoderma viride* (1,3-β-glucanase and 1,4-α-glucanase) were obtained from fermentation juice. The enzymic preparation was fractionated on a CM-Sephadex C50 column using a NaCl gradient in acetate buffer (3, 4).

The C.E.T. 1 and 2 were performed according to the following methodology :

C.E.T. 1 : 0.2 ml yeast cell suspension in buffer at 2.10^8 cells ml^{-1} ; 0.2 ml cystamine ClH 0.25 M in 0.1 M phosphate buffer, pH 6 ; 0.2 ml 1,3-β-glucanase (QM 806) 50 $u.ml^{-1}$.

C.E.T. 2 : 0.2 ml yeasts cells suspension in buffer at 2.10^8 cells ml^{-1} ; 0.2 ml 1,3-β-glucanase (*T. viride*) 3.4 $u.ml^{-1}$; 0.2 ml 1,4-α-glucanase (*T. viride*) 1 $u.ml^{-1}$.

In both test systems, the cells were osmotically stabilized by 0.6 M-KCl and incubated in hemolysis tubes on a reciprocal shaker at 26°C for 24 h.

Sphaeroplast formation was assessed by light microscopic examination as a function of cell burst on addition of water.

RESULTS.

The C.E.T. 1 allowed us to separate Ascomycetous from Basidiomycetous *Candida* as is shown in table 1. We repeated our experiments many times when possible comparatively to the collection species.

We tested the genus *Torulopsis* and our results are presented in table 2. We found that the main part of the species of these two genera had to be classified in Ascomycetes. However, 14 *Candida* and 4 *Torulopsis* are Basidiomycetous-like.

The results given by C.E.T. 2 are shown in table 3.

DISCUSSION.

The C.E.T. 1 and 2 seemed to be valuable taxonomic tests. We have shown (6) that the C.E.T. 1 (class test) is perfectly correlated with GC content which is considered as a good taxonomic criterion. We have localized the disruption site of the cell-wall (fig. 1 and 2) in *Saccharomyces cerevisiae* (7, 8) and we have found the same disruption site in *Candida albicans* as is shown in fig. 3 and 4. We think that this disruption site is strongly associated with the sexual reproduction and that it might be identical to the opening in the ascus-wall during the release of germinating ascospores (9). With C.E.T. 2, we have localized exactly the same disruption sites in basidiomycetous yeasts of the first sub-class. If we consider the structure of these disruption sites in both classes, we can observe a significant difference. In Ascomycetes, we know that the reducing agent acts before 1,3-β-

TABLE 1 Application of C.E.T. 1 (1,3-β-glucanase associated with a reducing agent) to separate ascomycetous from basidiomycetous *Candida*

	Ascomycetous *Candida*			
C.E.T.1 + *	*C. aaseri*	CBS 1913	*C. melinii*	CBS 1461
	albicans	IP 628	*membranaefaciens*	CBS 1952
	atmosphaerica	CBS 4547	*mesenterica*	CBS 602
	berthetii	CBS 5452	*norvegensis*	CBS 1922
	boidinii	CBS 3092	*obtusa*	CBS 1944
	brumptii	CBS 564	*parapsilosis*	CBS 604
	catenulata	CBS 565	*pelliculosa*	IP 606
	ciferrii	CBS 4856	*pseudotropicalis*	IP 513
	claussenii	CBS 1949	*pulcherrima*	IP 622
	cloacae	CBS 5612	*reukaufii*	CBS 611
	fabianii	CBS 5481	*rhagii*	CBS 4237
	guilliermondii	IP 47	*robusta*	IP 826
	ingens	CBS 4603	*rugosa*	IVP 27
	intermedia	CBS 572	*sake*	CBS 4094
	krusei	CBS 573	*silvae*	CBS 5498
	lambica	CBS 4550	*sloofii*	CBS 2429
	langeronii	CBS 1912	*solani*	CBS 1908
	lipolytica	CBS 599	*sorbosa*	CBS 1910
	lusitaniae	CBS 4413	*stellatoïdea*	CBS 1905
	macedoniensis	CBS 2079	*tenuis*	CBS 615
	maltosa	CBS 5611	*trigonopsoïdes*	CBS 1911
	melibiosica	CBS 5814	*tropicalis*	CBS 94
			truncata	CBS 1899
			utilis	CBS 621
			vanriji	CBS 2920
			viswanathii	CBS 4024
			zeylanoïdes	IP 207
	* Release of sphaeroplasts			
	Basidiomycetous *Candida*			
C.E.T.1 -	*aquatica*	CBS 5443	*humicola*	CBS 4280
	bogoriensis	CBS 4547	*japonica*	CBS 1906
	curvata	CBS 570	*javanica*	CBS 5236
	diffluens	CBS 5233	*marina*	CBS 5235
	foliarum	CBS 5234	*muscorum*	CBS 6921
	frigida (1)	CBS 5270	*nivalis* (1)	CBS 5266
	gelida (1)	CBS 5272	*scottii* (1)	CBS 614
	(1) Now classified in *Leucosporidium*			

TABLE 2 Application of C.E.T. 1 (1,3-β-glucanase associated with a reducing agent) to separate ascomycetous from basidiomycetous *Torulopsis*

	Ascomycetous *Torulopsis*			
C.E.T. + *	*T. anatomiae*	CBS 5247	*inconspicua*	CBS 180
	apis	CBS 2674	*magnoliae*	CBS 166
	bovina	CBS 2660	*maris*	CBS 5151
	candida	CBS 940	*molischiana*	CBS 136
	cantarellii	CBS 4878	*nitratophila*	CBS 2027
	colliculosa	CBS 133	*norvegica*	CBS 4239
	dattila	CBS 137	*pintolopesii*	CBS 1707
	ernobii	CBS 1737	*pinus*	CBS 970
	etchellsii	CBS 1751	*stellata*	CBS 843
	glabrata	CBS 138	*torresii*	CBS 5152
	globosa	CBS 162	*versatilis*	CBS 1731
	gropengiesseri	CBS 156	*wickerhamii*	CBS 2928
	holmii	CBS 135		
	* Release of sphaeroplasts			
	Basidiomycetous *Torulopsis*			
C.E.T. -	*T. fujisanensis*	CBS 4551		
	ingeniosa	CBS 4240		
	lactis-condensi	CBS 52		
	philyla	CBS 6272		

TABLE 3 Application of C.E.T. 2 (1,4-α-glucanase associated with 1,3-β-glucanase) to the basidiomycetous species of *Candida* and *Torulopsis*

C. E. T. 2 + *	C. E. T. 2 -
First sub-class	Second sub-class
Candida curvata	*Candida aquatica*
C. frigida	*C. bogoriensis*
C. gelida	*C. diffluens*
C. humicola	*C. foliarum*
C. japonica	*C. javanica*
C. marina	*C. muscorum*
C. nivalis	*Torulopsis fujisanensis*
C. scottii	*T. ingeniosa*
	T. lactis-condensi
	T. philyla
* Release of sphaeroplasts	

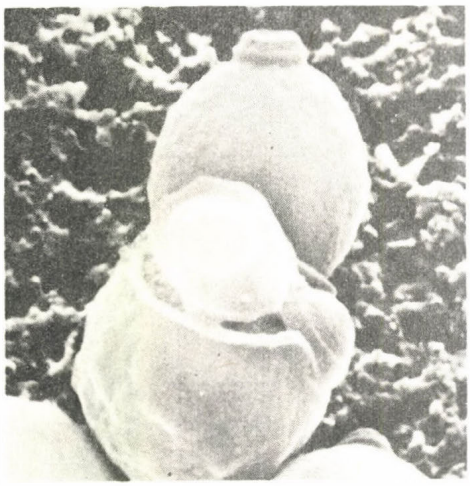


Fig. 1

Fig. 2

Fig. 1 and 2.- Release of sphaeroplast of *S. cerevisiae* using C.E.T. 1
Scanning electron microscopy x 15.000 (8)
(published in : Ann. Microbiol. Inst. Pasteur, 1979, in press)

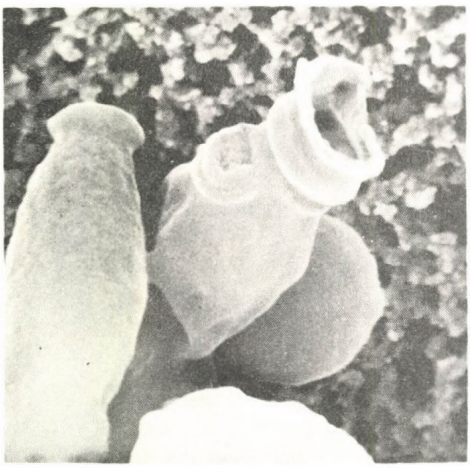


Fig. 3

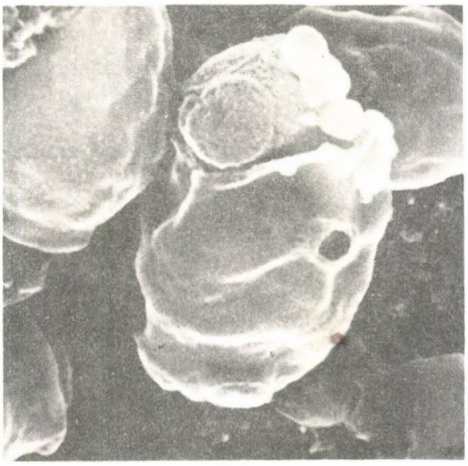
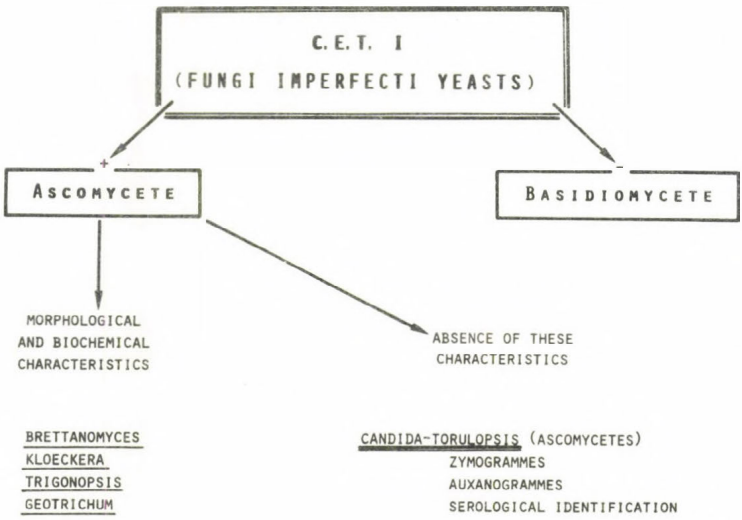
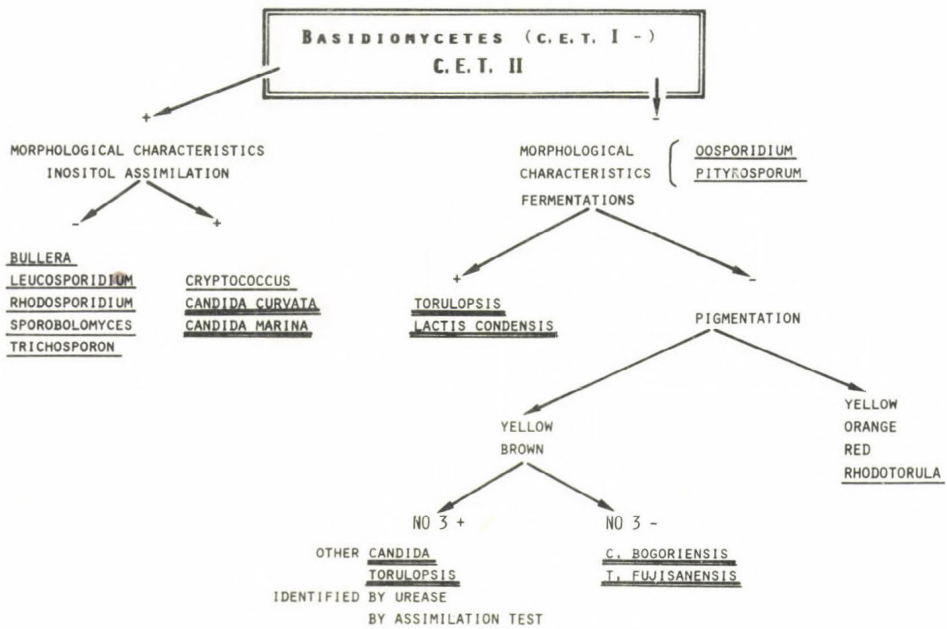


Fig. 4

Fig. 3 and 4.- Release of sphaeroplast of *C. albicans* using C.E.T. 1
Scanning electron microscopy x 15.000
We obtained these photographs with the collaboration
of M. Miegeville and C. Vermeil


C.E.T. I
(FUNGI IMPERFECTI YEASTS)
+
ASCOMYCETE
-
BASIDIOMYCETE
MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS
ABSENCE OF THESE CHARACTERISTICS
BRETTANOMYCES
KLOECKERA
TRIGONOPSIS
GEOTRICHUM
CANDIDA-TORULOPSIS (ASCOMYCETES)
ZYMOGRAMMES
AUXANOGRAMMES
SEROLOGICAL IDENTIFICATION


BASIDIOMYCETES (C.E.T. I -)
C.E.T. II
+
-
MORPHOLOGICAL CHARACTERISTICS
INOSITOL ASSIMILATION
-
+
BULLERA
LEUCOSPORIDIUM
RHODOSPORIDIUM
SPOROBOLOMYCES
TRICHOSPORON
CRYPTOCOCCUS
CANDIDA CURVATA
CANDIDA MARINA
MORPHOLOGICAL CHARACTERISTICS
FERMENTATIONS
OOSPORIDIUM
PITYROSPORUM
+
-
TORULOPSIS
LACTIS CONDENSIS
PIGMENTATION
YELLOW
BROWN
YELLOW
ORANGE
RED
RHODOTORULA
NO 3 +
NO 3 -
OTHER CANDIDA
TORULOPSIS
IDENTIFIED BY UREASE
BY ASSIMILATION TEST
C. BOGORIENSIS
T. FUJISANENSIS

glucanase. In sensitive Basidiomycetes, 1,4-α-glucanase acts before 1,3-β-glucanase. In both cases, the internal layer is made of 1,3-β-glucanes. The difference is localized in the external layer which is made up of protein with disulphide bonds in Ascomycetes, but is composed of 1,4-α-glucanes in sensitive Basidiomycetes. Starch is almost always synthesized by the latter.

The application of these C.E.T. tests to Fungi Imperfecti yeasts has led us to establish a new key (table 4) permitting a different placement of *Candida* and *Torulopsis* amongst the classification of yeasts.

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SPONTANEOUS AND ENZYME-INDUCED PROTOPLASTS FROM *ENTOMOPHTHORA* SPECIES

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ABSTRACT

A method was developed for the production of protoplasts from *Entomophthora virulenta*. Yields of up to 5 x 10^6 per ml were obtained using culture filtrates from *Trichoderma harzianum* grown on media supplemented with *E. virulenta* cell walls. The protoplasts were spherical, multinucleate, and readily regenerated to the normal hyphal form in nutrient media, including Grace's insect tissue culture medium. In this respect, they differ from *E. egressa* protoplasts, which will grow and multiply in the protoplast state in this medium.

In 1971, a species of *Entomophthora* was isolated from naturally-infected hemlock looper (*Lambdina fiscellaria fiscellaria*) larvae collected in Newfoundland (1). Experiments designed to improve the growth of this isolate revealed that the fungus possessed the unusual property, when its conidia germinated in Grace's insect tissue culture medium (2) supplemented with fetal bovine serum, of spontaneously releasing the protoplast from the tip of the conidial germ tube (3). The same phenomenon of protoplast release was observed if vegetative hyphae were transferred to this medium.

Upon release, the protoplast rapidly organised into spindle-shaped cells joined at each end by fine thread-like processes. Subsequently, they readily divide and multiply, and can be maintained in the protoplast state by periodic subculture in the same medium.

In shake culture, the protoplasts grow predominantly as individual cells rather than in chains of cells, and will undergo cell wall regeneration, hyphal formation and ultimately conidial formation (4, 5, 6).

Lepidopterous larvae can readily be infected by injection of a protoplast suspension into the hemocoele, and further evidence that the protoplast phase represents part of the natural cycle of the fungus was obtained when osmotically-fragile bodies were found in the hemolymph of laboratory-infected spruce budworm (*Choristoneura fumiferana*) larvae (7).

Since 1971, 15 more strains of the same species have been isolated from other Lepidoptera larvae (8) and *E. grylli* has also been isolated from

infected Carolina grasshoppers (*Dissosteira carolina*), and grown in protoplast form (9).

Numerous attempts to obtain protoplasts by similar means from other *Entomophthora* species have been unsuccessful, although the technique has formed the basis of a useful method for isolation of *Entomophthora* species in pure culture (10).

In order to gain a clearer understanding of the similarities and differences between the *E. egressa* and *E. grylli* protoplasts and those of other *Entomophthora* species, the production of protoplasts from *Entomophthora* species by conventional enzymic techniques has been studied. The relevance of these experiments is enhanced by recent advances in fungal hybridization via protoplast fusion (11), where such techniques may be used to improve pathogenicity, ease of mass production, or other desirable characteristics, and so facilitate the use of *Entomophthora* species in biological control programmes.

The present paper reports the production of protoplasts from *E. virulenta*, and compares their properties with those of *E. egressa*.

MATERIALS AND METHODS

E. egressa strain 458 and *E. virulenta* strain FP5 were obtained from the FPMI collection, Sault Ste. Marie. *E. egressa* was maintained by periodic subculture in Grace's insect tissue culture medium (2) supplemented with 5% (v/v) fetal bovine serum (FBS) while *E. virulenta* was maintained on Sabouraud dextrose-milk-egg yolk medium (12). *Trichoderma harzianum* CBS 354-33 was obtained from Prof. J.H.G. Wessels and maintained on TLE medium supplemented with 1% laminaria meal and 0.5% chitin (13).

Cell walls of *E. virulenta* were obtained by disruption of 4 day old shake-grown mycelium with 0.5 mm diameter glass beads, followed by extensive washing with distilled water. Cell walls (5 g/l) were added to TLE medium (500ml per 2 l conical flask), and inoculated with *T. harzianum* conidia (final concentration of conidia, 1×10^6 ml^{-1}). The flasks were incubated on a rotary shaker (200 cycles min^{-1}) at 28° until the walls were digested (about 3 days), when the mycelium was removed and the culture fluid concentrated by rotary evaporation under reduced pressure at 30° or by freeze drying.

E. virulenta mycelia for protoplast release studies were grown in glucose (2%) peptone (2%), yeast extract (0.1%) medium on a rotary shaker (150 cycles min^{-1}) at 23°. The mycelium was washed three times in sterile distilled water and once in 0.6M stabilizer. Known fresh weights of mycelium were added to equal volumes of lytic enzyme preparation and incubated at 28° on a reciprocal shaker.

RESULTS AND DISCUSSION

Initial attempts to obtain protoplasts from *E. virulenta* hyphal bodies using concentrated culture filtrates from *T. harzianum* grown on TLE medium supplemented with chitin and laminaria meal (13) were unsuccessful. Washed *E. virulenta* cell walls were therefore used as lytic enzyme inducing agent. When incorporated into the TLE medium (0.5%, w/v), they were rapidly digested by *T. harzianum* and culture filtrates concentrated

by either rotary evaporation or freeze-drying readily produced protoplasts from *E. virulenta* in 0.6M KCl stabilizer at pH5.8. No protoplasts were formed if 0.6M $MgSO_4$ was substituted as stabilizer. While both rotary-evaporated and freeze-dried preparation had the same efficiency when freshly-prepared, the freeze-dried preparation gradually lost efficiency, and two to three months at -20° was the maximum storage period for this preparation. The rotary-evaporated preparation, however, retained its efficiency for at least 8 months under the same storage conditions. Figure 1 shows a typical protoplast release curve for *E. virulenta* using freeze-dried enzyme.

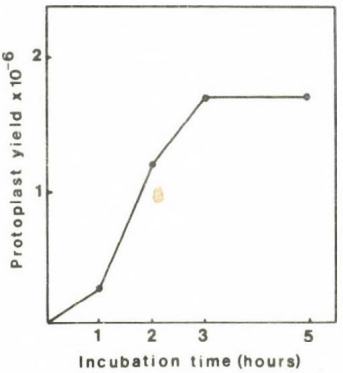


Fig. 1. Effect of time on protoplast release from *E. virulenta*. The digestion mixture contained 20mg ml^{-1} fresh weight mycelium and *T. harzianum* freeze-dried culture filtrate reconstituted at 5x original concentration in 0.6M KCl/0.2M phosphate buffer stabilizer pH 5.8.

E. virulenta protoplasts are spherical, averaging 5.5 µm in diameter, multi-nucleate, and similar in appearance to protoplasts prepared in the same manner from other fungal species (Figure 2).

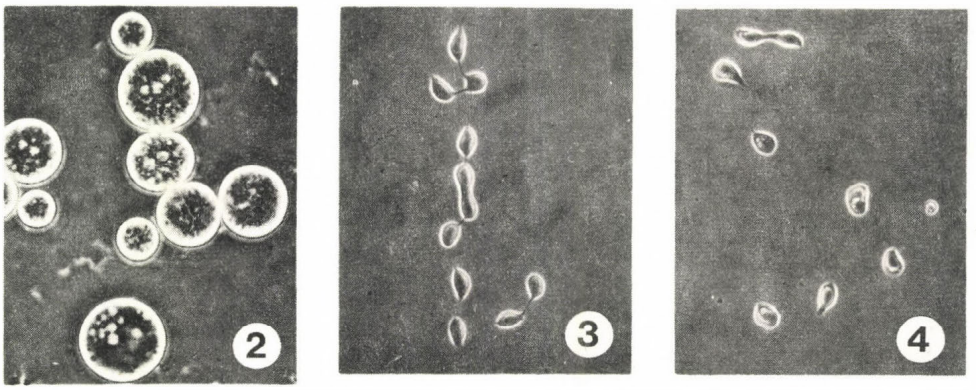


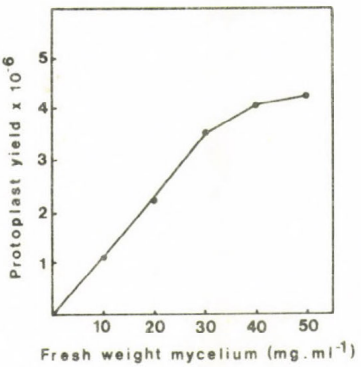
Fig. 2. Protoplasts of *E. virulenta*. (3 hour digestion; conditions as in Figure 1).

Fig. 3. Protoplasts of *E. egressa* is still culture. (48 hours incubation; Graces insect tissue culture medium plus 5% FBS).

Fig. 4. Protoplasts of *E. egressa* in shake culture (24 hours incubation; medium as in Fig. 3).

In contrast, the morphology of *E. egressa* protoplasts from still (Figure 3), and shake (Figure 4) culture are substantially different, although they do assume a spherical form in 0.6M KCl stabilizer, reverting to the spindle shape as growth recommences after transfer back to Grace's medium. *E. virulenta* protoplasts, on the other hand, remain spherical in Grace's medium, and regenerate to the hyphal form within 12 hours.

The relationship between fresh weight of mycelium and protoplast release is shown in Figure 5, and the effect of age of mycelium on protoplast release is shown in Figure 6. The growth curve of *E. virulenta* is also included in this figure. Maximum yields of protoplasts were obtained from mycelium in the stationary and decline phases of the growth cycle, although this may be because the enzyme was raised against 4-day-old mycelium, and wall composition may change with age.



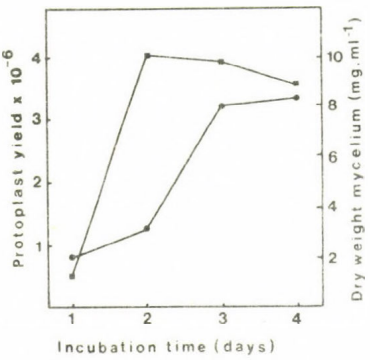
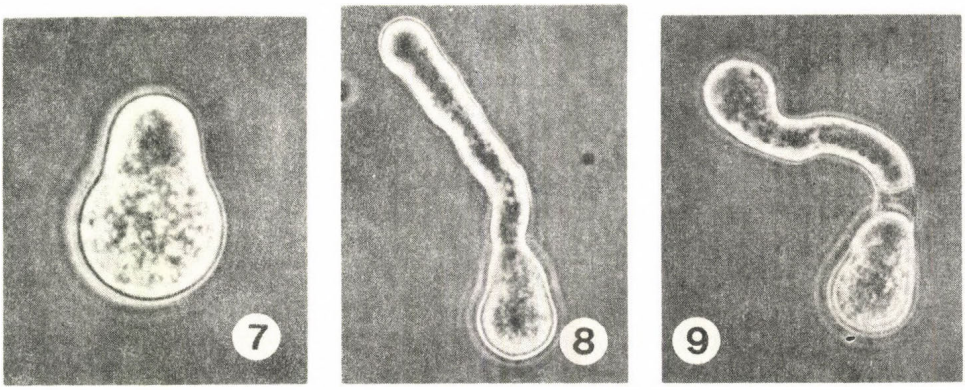


Fig. 5. Relationship of fresh weight of mycelium to protoplast release in *E. virulenta*. Digestion conditions were as in Fig. 1, except that the fresh weight of mycelium was varied. Protoplast yield was counted after 5 hours digestion.

Fig. 6. Effect of age of mycelium on protoplast yield (● ——— ●) and growth curve (■ ——— ■) of *E. virulenta*. The fungus was grown in shake culture at 23°. Samples were taken at 24 hour intervals, washed, and the dry weight determined. Parallel samples were digested as described in Fig. 1 and protoplast yield determined after 5 hours.

When the lytic mixture is removed and replaced by stabilized nutrient media, *E. virulenta* protoplasts readily regenerate to give the normal hyphal form (Figures 5, 6 and 7). These developing hyphal bodies can then be transferred to unstabilized media, and normal development occurs.



Figs. 7, 8, 9. Regeneration of *E. virulenta* protoplasts in 0.6M KCl stabilized glucose-peptone-yeast extract medium.

The experiments outlined above demonstrate that protoplasts can readily be obtained from *E. virulenta* by lytic enzyme techniques, and that subsequent regeneration of these protoplasts is similar to that described for other fungi. That this regeneration sequence is also followed in Grace's medium indicates that the ability of *E. egressa* protoplasts to grow and multiply in that medium is not shared by *E. virulenta*, and furthermore, this failure is not caused by the inability of the latter to release its protoplast spontaneously when incubated in Grace's medium.

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Fusion of Micromonospora protoplasts

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Most actinomycetes produce antibiotics. *Micromonospora* species, which also belongs to actinomycetes, has recently emerged as a producer of important antibiotics, like gentamicin, (1) sisomicin (2), etc. The mechanism and regulation of antibiotic synthesis can be investigated by biochemical and genetical methods (3). Genetic investigation needs the development of a good genetic transfer method. In case of *Micromonospora*, however, only a poorly characterized conjugation type of genetic exchange has been described (4, 5). It has recently been demonstrated that very efficient recombination can be obtained by fusing of bacterial protoplasts with polyethylene glycol treatment (6, 7). This method has been applied for several streptomycetes succesfully (8). Since polyethylene glycol (PEG) is able to fuse practically any kind of protoplasts as well as to induce heterospecific crosses it seemed us reasonable to try to develop an effective protoplast fusion system with *Micromonospora*.

MATERIALS AND METHODS

Two species of *Micromonospora* were used throughout this study. *Micromonospora echinospora* ATCC 15837 which synthesizes gentamicin complex (1) and *M. inyoensis* ATCC 27600 which produces the antibiotic sisomicin (2). They were cultured in a rich medium or minimal medium (9) at 32°C. Antibiotic resistant mutants were obtained after nitrosoguanidine mutagenesis by plating onto antibiotic containing solid medium. The rifampicin resistant mutant (*rif*^{r}) could grow in the presence of 100 µg/ml of rifampicin, whereas the wild type parent was very sensitive for this drug (less than 1 µg/ml resulted in complete growth inhibition). The same parameters for streptomycin resistant mutant were 500 µg/ml and 10 µg/ml respectively. Casamino acid-dependent mutant (*cas*) was isolated after enrichment with penicillin-cycloserine treatment by replica-plating. The amino acid auxotrophy of the mutant could not be determined, since it did not grow on mineral salts-glucose medium supplemented with either of

the amino acid alone, and *vice versa*, good growth was obtained in the absence of any amino acid if the other 19 natural amino acids were supplemented. The mutant reverted by the frequency of less than 10^{-7} and did not give a background growth on minimal-glucose medium. This latter property was very important when direct selection was used after fusion.

RESULTS AND DISCUSSION

Protoplasting. *Micromonospora* could not be effectively protoplasted with lysozyme or lysozyme-EDTA treatment even if very high concentrations of lysozyme were used (up to 20 mg/ml) for 24 hours. However, when the cells were grown in the presence of high concentration of glycine, 1 mg/ml of lysozyme was enough for complete protoplast conversion in 90 min at 37°C. *Micromonospora* was found to be much more sensitive for glycine than the streptomycetes (10), since 0.5% of glycine inhibited very severely the growth (Fig. 1). The glycine sensitivity of various *Micromonospora* strains were different, therefore it was necessary to determine the optimal concentration of glycine not only with every strain but also with every mutant. Good protoplasts were obtained only if the morphological changes shown in Fig. 2 were observed under phase contrast microscope. In case of wild type, 0.3% glycine induced the characteristic morphological changes, whereas in case of *cas rif*^{r} double mutant 0.2% glycine was enough. Under optimal condition the protoplast conversion was almost complete, *i.e.* the colony forming ability decreased after lysozyme treatment to 10^{-7} - 10^{-8}.

Regeneration of protoplasts. A modified method of Okanishi (9) was used for cell wall regeneration of *Micromonospora* protoplasts. Table 1 demonstrates the essential modifications.

TABLE 1 Medium for regeneration of *Micromonospora* protoplasts

Components	Okanishi	Modified
Sucrose	0.35 M	0.2 M
K_2SO_4	0.25 g/l	0.25 g/l
Glucose	1.0%	1.0%
Proline	0.3%	0.3%
Casamino acid	0.1%	-
$MgCl_2$	50 mM	50 mM
$CaCl_2$	20 mM	10 mM
KH_2PO_4	0.36 mM	0.5 mM
Buffer	0.025 M TES	0.025 M TRIS
Temperature	28°C	32°C
pH	pH 7.2	pH 7.5

As can be seen 0.2 M sucrose was used instead of 0.35 M, because the latter concentration was found to be inhibitory. Casamino acid was removed from the regeneration medium to make

possible the direct selection of recombinants after fusion. When indirect selection was used, the medium was supplemented with 0.1% of yeast extract instead of casamino acid which resulted better and more rapid regeneration. To change the temperature from 28°C to 32°C was important since it increased the regeneration frequency by a magnitude. More important was to increase the pH value from pH 7.2 to pH 7.5. This change resulted in a further increase of the regeneration frequency by a factor of 100. By these modification altogether, the efficiency of regeneration could be improved considerably, the difference was higher than 10^3. Under optimal condition the regeneration frequency was around 10^{-3}.

Fusion of protoplasts. The protoplasts obtained from the mutant and the wild type strains were fused with polyethylene glycol (PEG) treatment as described already (6). Briefly, 40% PEG 6000 was used for 10 min at room temperature. 0.1 ml of the fused protoplasts were than plated in 2 ml osmotically buffered top agar (0.4%) onto regeneration media.

Selection of recombinants. In principle two kinds of recombinant selection method can be applied after protoplast fusion, *i.e.* direct (6) and indirect (7) selection. When direct selection is used, the PEG-treated protoplasts are plated onto selective medium, where only the recombinants are able to form colonies. In case of indirect recombinant selection method, the fused protoplasts first are allowed to regenerate on a nonselective medium and the recombinants are selected by replica plating onto selective medium. Both methods were used for selecting recombinants after fusion of different *Micromonospora* protoplasts.

Indirect method. Protoplasts of the *cas* rif^r double mutant and its wild type (*cas*$^+$ rif^s) were used for indirect selection. After fusion protoplasts were plated onto regeneration medium described in Table 1 which contained 0.1% yeast extract too. After the colonies had been developed, they were replica-plated onto the same as well as onto selective medium (minimal medium containing 5 µg/ml of rifampicin). By these methods the frequency of hybrid colonies (prototrophic-rifampicin resistant) was found to be around 10^{-4}. Most of them, however, proved to be unstable, less than 1% showed stable recombinant properties. Thus the final recombination frequency was very low. No colony was formed at all when the fused protoplasts were plated directly onto selective medium indicating that the rif^r property, as expected, is recessive in *Micromonospora* too. Among the unstable hybrids some interesting ones were found. Although we could reisolate both parents from a single colony, no growth was observed on the selective medium. The explanation of this phenomenon is not known but it seems as if they were heterokaryons.

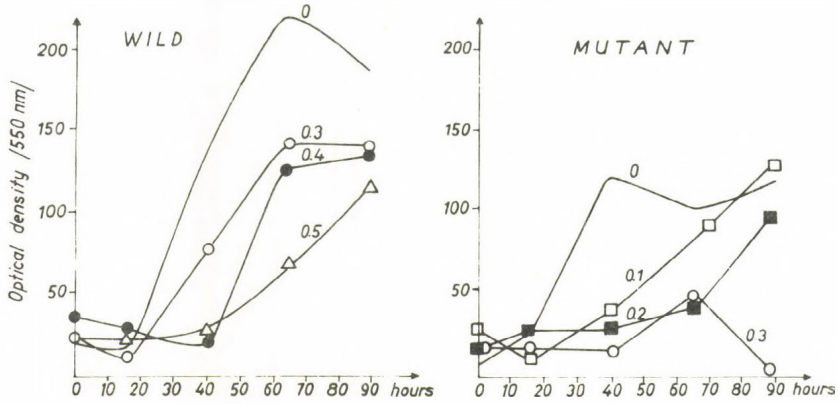

Fig. 1 Effect of glycine on growth of *M. inyoensis*

Numbers on the curves indicate concentrations of glycine in percent.

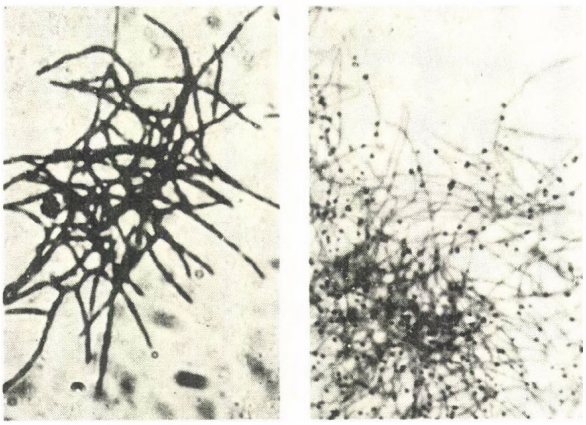

Fig. 2 Morphological changes of a *M. inyoensis* culture induced by glycine.

A: without glycine
B: with 0.3% glycine

Direct selection. Since the mutations for antibiotic resistance are normally recessive ones with respect to their wild type alleles and after protoplast fusion most probably an intensive cytoplasmic mixing takes place, it is not surprising that direct selection can be used only if the selective antibiotic is added to the regeneration medium after waiting the phenotypic lag. For this experiment we used the protoplasts derived from the wild type *M. echinospora* and from its *cas* str^r double mutant. The fused protoplasts were plated onto regeneration medium given in Table 1. (The concentrations of Ca and Mg were decreased to 10 mM, since the high concentrations of these cations are known to counteract the streptomycin effect.) Then streptomycin was over-layered in osmotically buffered top-agar either immediately after plating or one, two, etc., days later, to give a final concentration of 200-400 µg/ml. No colony was found on those Petri dishes which were supplemented with streptomycin on the first two days. When streptomycin was added between the third and seventh days after plating, hybrid colonies developed. More than 90% of them proved to be stable recombinants (good growth in five successive passages on minimal-streptomycin medium).

Fusion of heat-inactivated and living protoplasts. It is apparent from the results described above that both the direct and the indirect method has its own advantage and disadvantage. Advantage of the direct selection is that recombinants can be obtained in one step, whereas in the latter case in two steps. In addition, the vast majority of the directly isolated hybrids proved to be stable recombinants but more than 99% of the colonies isolated as "recombinants" by replica plating were found to be unstable hybrids. On the other hand, the indirect selection has the advantage that it gives a better and more rapid cell wall regeneration as well as the antibiotic resistant markers can also be used for selection without any laborious overlayering and waiting for phenotypic lag.
To circumvent the abovementioned disadvantages of both techniques we concentrated our efforts to develop a simpler system for recombinant selection. The requirements for this system are the following: *i*, to use the direct recombinant selection technique without using antibiotic resistant markers; *ii*, to avoid the isolation of more than one auxotrophic mutant since it is not too easy to isolate them from *Micromonospora*. A new technique has recently been devised in the Alföldi's laboratory (11) for direct selection of recombinants after fusion of *Bacillus megaterium* protoplasts. In this technique protoplasts of one parent are inactivated by heat treatment prior to fusion. This procedure provides a means for counterselection when a prototrophic parent is used. The heat treatment should be effective enough to kill all the protoplasts but, at the same time, should be mild enough not to lyse them. It was found that this technique could be used for *Micromonospora* too. The protoplasts of wild type parent was heat inactivated by incubating them at 55°C for 2-3 hours before fusion. The other parent was the *cas* str^r double mutant. After PEG treatment

the fused protoplasts were layered onto minimal medium, where only the recombinants were able to form colonies, since neither the heat-inactivated prototrophic parent, nor the other parent (*cas* str^r) alone formed colonies at all on this medium. Furthermore, about 80% of the hybrids isolated by this method was found to be streptomycin resistant. Since the antibiotic production is known to be decreased concommitantly with some auxotrophic mutations, the advantage of a direct selection system which needs only one genetically marked parent is evident.

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PLANT PROTOPLASTS

THE OLD PROBLEM OF PROTOPLAST CULTURE: CEREALS

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On the basis of 6 years working, exclusive on cereal protoplast culture with hundreds of genotypes, and from discussions and literature study we are convinced that this problem can be solved, if given enough support and attention. We identify and recommend those systems which, at present, are the most promising.

Why cereal protoplasts?

About 80 % of human food is provided, directly or indirectly, by cereals and grasses (1). If protoplast technology - plant regeneration/genetic manipulation/genetic engineering - will in any way contribute to an improvement of plants, such an improvement, therefore, might have its greatest effect in the graminaceous crop plants. Routine plant regeneration from protoplasts is the basic prerequisite for an effective application of the rapidly developing techniques in genetic modification of higher plant cells. Therefore, since the onset of modern protoplast research numerous workers have invested all their efforts in experiments aimed at the development of methods for the induction of sustained divisions and plant regeneration in cereals and grasses. However, despite an immense effort, success, especially if compared with herbaceous dicot plant species, is depressingly low. Since the successful work of Vasil and Vasil, who were able to establish an embryogenic suspension culture of Pennisetum americanum from which protoplasts could be isolated which again formed embryos and plants (V. and I. Vasil, personal comm.), and from the early work of Gamborg et al. (2), who established an embryogenic culture of Bromus inermis, and of Kao et al. (3), who recovered embryos and plants from protoplasts isolated from this culture, we may conclude that, given enough patience, effort, and support, it will, one day, be possible to grow cereals from single cells, as readily as in, e.g., tobacco. So far, however, we still seem to be far from this goal. We hope, however, that the results with Bromus and Pennisetum can be repeated with the main cereals like wheat, rice, maize, and barley. The present state of the art with cereals and grasses is, to my understanding, as follows:

The few dividing systems

Table 1 Cereal protoplast culture:state of the art(June 1979)

1. Plant regeneration from isolated protoplasts
   Bromus inermis (not reproduced): Kao, K.N. et al., 1973 (3)
   Pennisetum americanum: Vasil & Vasil, pers. comm.

2. Cell cultures established from protoplasts isolated from plant organs
   Oryza sativa (not reproduced): Deka & Sen, 1976 (4)
   Zea mays (not reproduced): Potrykus, I. et al., 1977 (5)

3. Cell cultures established from protoplasts isolated from apparently non-morphogenetic cell cultures
   Saccharum officinale: Maretzki & Nickell, 1973 (6)
   Oryza sativa: Somatic hybrid. Group, Peking, 1975 (7)
   Hordeum vulgare: Koblitz, H., 1976 (8)
   Triticum monococcum: Dudits, D., 1976 (9)
   Oryza sativa: Deka & Sen, 1976 (4)
   Oryza sativa: Chi-kuei, T. et al., 1978 (10)
   Zea mays: Potrykus, I. et al., 1979 (11)
   Pennisetum ssp.: Vasil & Vasil, pers. comm.
   Sorghum bicolor: Brar, D.S. et al., in press (12)

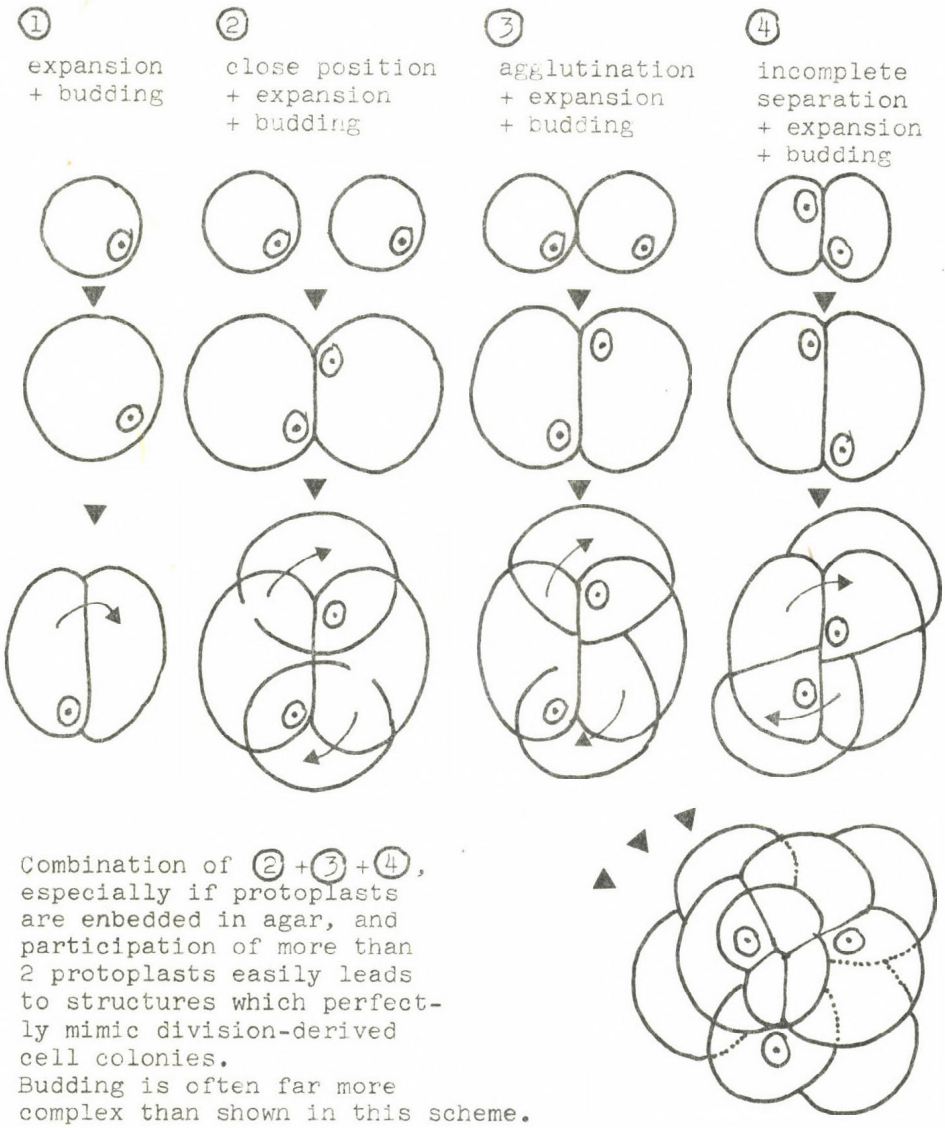
4. Reports where division ceased rapidly or where division has not been demonstrated convincingly
   Numerous: see references (13) to (47)

5. Divisions of cereal nuclei within interspecific fusion hybrids
   Soybean+barley, soybean+corn: Kao, K.N. et al., 1974 (46)
   Carrot+barley: Dudits, D. et al., 1976 (47)

6. Division of cereal nuclei without subsequent cell division
   Avena sativa: Galston, A.W., pers. comm.
   Hordeum vulgare, Triticum aestivum: Lörz, H., pers. comm.
   Zea mays: Potrykus, I., unpublished

The problem of misinterpretation: There are numerous reports in the literature on cereal protoplasts culture, where figures, published to demonstrate divisions, apparently indicate misinterpretations of developmental stages which often occur in cereal protoplast cultures and which perfectly resemble true divisions but which, unfortunately, are artifacts (see Section 4, Table 1). Cereal protoplasts very often respond in culture with complex budding structures which remarkably resemble first and second divisions. If protoplasts are plated into soft agar, close location in combination with the normal expansion of the protoplasts also leads to perfect "division-like" structures. Agglutination and expansion, incomplete separation during isolation (frequent in preparations from more meristematic tissues) as well as internal septation of spontaneous fusion products are further possible sources for developments which mimic cell colony formation from protoplasts. Combination of budding, expansion, incomplete separation, agglutination, close location in agar also leads to

Fig. 1: Pseudo-divisions in cereal protoplast cultures: some of the possible causes for the misinterpreting of "divisions"



structures which, without having undergone a single division, resemble cell clusters derived via several rounds of divisions. This is especially complex in cases where more than 2 protoplasts contribute to the structure (see Fig. 1). Experience with dividing protoplast systems together with precise control of the protoplast populations and careful observation during

their development may help in avoiding misinterpretation. A further source for possible errors, relatively common in protoplast preparations from meristematic and from expanding tissues and cell cultures, are groups of meristems which "contaminate" protoplast populations if these are not cleaned carefully.

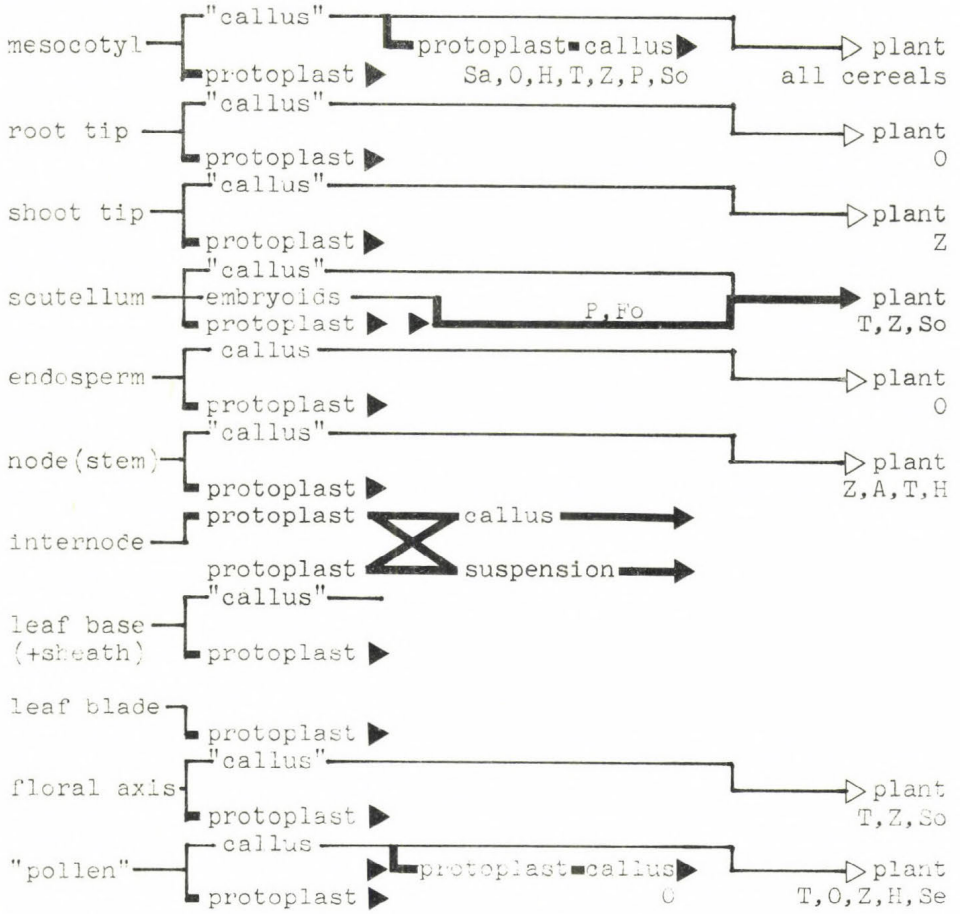
Present situation in cereal protoplast/"tissue" culture

Cereal protoplasts do not respond to the wide range of conditions inducing division of protoplasts from herbaceous dicotyledoneous species and of Asparagus (48). Despite the application of the large body of experience accumulated in more than 10 years of successful (and unsuccessful) culture of plant protoplasts, and despite the testing of hundreds or thousands of in vitro conditions (31-39), we are not in the position to induce sustained divisions at will in any given cereal. The situation is even more serious with regard to plant regeneration. The embryogenic cell culture (2) and protoplast culture (3) of Bromus was, probably, a chance event. We do not yet know whether the embryogenic Pennisetum culture of Vasil & Vasil will also be unique (although we consider it a very exciting improvement). Both the results with Oryza sativa (4) and Zea mays (5), where protoplasts isolated directly from the differentiated plant formed cell cultures, were uncontrollable chance events. The apparent non-morphogenetic cell lines of Saccharum off. (6), Triticum mon. (9), Zea mays(11), Oryza sat.(4), Pennisetum spp. (Vasil, pers. comm.), and Sorghum bic. (12) have been selected as chance events and, as they are not morphogenic to begin with there is not much hope that they might yield morphogenic protoplasts. The anther culture derived cell cultures of Oryza sativa (7,10) may have been morphogenetic at the beginning but had lost their morphogenic potential at the time of the protoplast experiments. The situation with cereal mesophyll protoplasts, I consider, as far as published, rather negative. Most of the published "first divisions" are, probably, misinterpretations of "pseudo-divisions". However, there are some rare figures, which might show real first divisions (23, 25). In two publications it has been demonstrated that cereal mesophyll nuclei can enter mitosis if combined with an actively dividing nucleus in a heterokaryon (46, 47), and there are several observations on the division of nuclei within cultured cereal mesophyll protoplasts which indicate that nuclei are not completely blocked towards division (Section 6, Table 1). Under appropriate culture conditions cereal mesophyll protoplasts can survive more than 120 days in culture and can be induced to develop highly synchronously towards division, showing all the well-known intracellular alterations characteristic for mesophyll protoplasts of, e. g., tobacco developing towards division (32,33,34). The further development, however, ends in pseudodivisions.

The problem of cereal protoplast culture appears not to be a problem of cereal protoplasts but rather a problem of cereal cell culture (49,50). Induction of cell cultures in cereals apparently leads to a totally different pattern of de-

velopment than in herbaceous dicots. Whereas dicots respond with a typical callus formation - proliferating, undifferentiated masses of cells each of which is capable of sustained divisions - cereals apparently respond with the development of localised adventitious bud or root primordia which contain only a few cells capable of sustained divisions and which produce masses of terminally differentiated cells (49,51,52). Cell line formation (53) - development of exclusively proliferating and undifferentiated cells - is an uncontrollable and

Fig. 2: "Tissue" and protoplast culture in cereals and grasses



T = Triticum A = Avena
O = Oryza So= Sorghum
Z = Zea mays P = Pennisetum
H = Hordeum Fo= Forage grass
Se= Secale Sa= Saccharum

"callus" = callus-like masses of non-dividing cells and proliferating groups of organised meristematic centers. Plant regeneration not from single cells.

rare chance event which is, in addition, extremely species and genotype dependent (49). Plant regeneration from cereal "calluses" is, apparently, only from preexisting (and propagating) organised structures, not from undifferentiated single cells (see Fig. 2).

With regard to our aim - plant regeneration from isolated single cells/protoplasts in cereals - there are some promising systems in cereal cell culture: 1. Microspore-derived cell cultures clearly originate from single cells. However, multiple plant formation from anther culture derived cell cultures may arrive through the typical cereal culture response of modified original embryo. 2. Multiple plant regeneration from scutellum tissue of immature embryos (54,55,56) appears to be through somatic embryogenesis originating from single cells (57), and might be developed into a source of embryogenic cell cultures similar to Daucus carota. 3. Plant regeneration from cell cultures derived from cultured roots of Oryza sativa (58) indicates complete de- and re-differentiation. 4. Plant regeneration from the shoot system of immature inflorescences (56, 59,60,61) may be from dormant axilliary bud initials. It might, however, also arrive from single undifferentiated cells and deserves a detailed study. 5. Plant regeneration from endosperm (62) ought to be from single cell origin and would provide an interesting protoplast system.

How to proceed further?

A basic difference between cereals/grasses and the herbaceous dicots, where cell culture is routinely possible, is the "wound-reaction". Induction of sustained divisions in dicots is, possibly, not more than supporting the naturally occurring process of wound healing at the single cell level, and, therefore, is not really an "induction". It is a common experience that in all tissue culture approaches, we are totally dependent upon the reaction of "competent" cells and we have no detailed information about the mechanisms underlying and regulating this phenomenon. The wound-reaction may be indicative of competence for in vitro response. Differentiated cereal cells do not show a wound reaction.

It might, therefore, be advisable to focus the work on cereal protoplast culture in future on cells which have expressed division potential, and better, if possible, on cells which have expressed totipotency in complex cell culture systems. There are, if compared to herbaceous dicots, not many cells in a cereal/grass which fulfil this requirement , however, there are at least some cells and tissues, which, under this view, deserve more attention: Totipotent cells are available 1. in "induced" microspores and in microspore-derived cell cultures (before they have lost their regeneration capacity), 2. in the scutella of "induced" immature embryos at the state when secondary embryos are formed (when small plants become visible it is probably already too late), 3. in the immature florescences at the beginning of multiple shoot formation (it has not yet been established where exactly the responding cells are located), 4. in explanted and "induced" vegetative growing points, 5. in the immature, cellular endosperm. It is, with the exception of

the microspores, so far unknown which cells and how many of these cells are totipotent and, therefore, protoplast populations isolated from these tissues will, probably, be composed of mixtures of competent and non-competent cells. It may, therefore, be advisable to at first approach a multiplication of competent cells by trying to establish an embryogenic cell culture prior to protoplast isolation. Cells having division potential, however, without experimentally demonstrated totipotency, are present in meristematic leaves and young leaf sheath, as has been, among others, demonstrated by Koblitz and co-workers (63, 64,65), and in roots, nodes, and axillary buds. Protoplasts from roots have, however, with the exception of an unconvincing claim (42) never been induced to undergo sustained divisions.

From the rich experience based on protoplast culture experiments with the numerous positively responding dicots we know that many groups of parameters control success or failure of a protoplast culture experiment:

Table 2 Some of the parameters which control protoplast division and subsequent plant regeneration

1	genetic basis for in vitro competence
2	intercellular relationships within the plant
3	mechanisms of differentiation (ontogeny)
4	history of the individual plant/organ
5	actual physiological state of the cell
6	effects of the isolation procedure
7	effects of the isolated single cell state
8	nutritional requirements
9	hormonal requirements
10	physical culture conditions
11	presumptive inhibitors
12	cell population density effects
13	metabolism of cell wall synthesis
14	regulation of cell wall synthesis
15	function of cell wall in cell division
16	regulation of dedifferentiation
17	regulation of nuclear division
18	regulation of cellular division
19	regulation of cellular differentiation
20	intercellular relations within cell cultures
21	regulation and mechanism of pattern formation
22	regulation of organ/embryo formation

Unfortunately we know little more about these parameters than that they may be critical, as not many studies with model plant protoplast systems have aimed at providing more basic information upon the mechanisms underlying and regulating the events finally leading to protoplast-derived plants. As we know, however, that each of these factors alone can be the limiting factor, we have to consider them all in the cereal

protoplast culture experiments.

Cereal protoplasts require more attention

If we hope that protoplast technology might be developed into a functional method for stable genetic alteration (and hopefully improvement) of plants, and if we do not close our eyes before the serious problem of food production, and if we consider that the present high yield of crop plants is only possible on the basis of an enormous energy input, and if we hope that, e.g. the progress in the molecular analysis of the nitrogen fixing systems and in recombinant DNA research and in transformation (66) might flow into a successful transfer of nitrogen-fixing capacity into plants, and if we do not want this to be possible with rather useless model plants and not possible with those plants which produce 4/5 of human food, then we have now to undertake a serious effort to crack the cereal protoplast problem.

It is apparent that cereals are extremely difficult in this respect and that Solanaceous species respond very readily. It is understandable therefore, that the great majority prefers to work with Solanaceae and only few work with cereals. As we miss, apparently, some basic knowledge upon why cereals in general are so unresponsive, the cereal in vitro problem needs the attetion of as many researchers as ever possible to attack this problem from as many angles and levels as possible and with as many genotypes as possible. Work with responding model plants should try to put more emphasis onto experiments which would allow us to find out how the many parameters, which influence the sequence of events which leads to the recovery of a plant from a protoplast, interfere with, support or regulate this sequence.

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EMBRYOGENESIS AND PLANTLET FORMATION FROM PROTOPLASTS OF PEARL MILLET (PENNISETUM AMERICANUM)

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ABSTRACT

The culture of cereal protoplasts has proved to be extremely difficult, and there are no reports of embryoid, shoot or plantlet formation from cereal protoplasts. Callus tissue was obtained from hypocotyls of young seedlings of pearl millet (Pennisetum americanum). A rapidly growing cell line was initiated from these callus cultures in a liquid nutrient medium in suspension culture. Protoplasts isolated from suspension culture cells had a plating efficiency of 15-20%, and gave rise to callus tissues which could be sub-cultured. Attempts to induce organogenesis in these protoplast-derived callus cultures have not been successful. Therefore, immature embryos of pearl millet were cultured to obtain fresh callus tissues, and new suspension cultures were initiated from them. These suspension cultures are embryogenic, and on plating form embryoids, shoots, roots, and plantlets. Protoplasts isolated from suspension cultures of immature embryo callus also regenerate cell walls and give rise to cell masses after sustained cell divisions. Upon transfer to a hormone-free medium, plantlets are formed through the process of somatic embryogenesis. This first demonstration of plantlet formation from cereal protoplasts should encourage similar efforts in other cereal species.

INTRODUCTION

The potential of plant protoplast research in crop improvement and genetic engineering has been extensively discussed in recent years (1,2). Although remarkable progress has indeed been made in the use of this technology to obtain somatic hybrid plants in some cases (3,4,5,6), the fact remains that such success is still limited to model plant systems like Datura, Daucus, Nicotiana, Petunia, etc., and in most of these cases sexual hybridization is known to occur. The techniques of somatic hybridization by protoplast fusion, and the possibility of genetic modification by the induced uptake of cell organelles, plasmids, DNA, etc., can not yet be applied to cereals and legumes - the two most important groups of plants for man and his domesticated animals - because even the basic techniques for the culture of their protoplasts are not currently available. The culture of cereal protoplasts has proved to be particularly difficult, as shown by the following two reports:

"Intensive efforts to explore the conditions which induce sustained cellular divisions in cereal leaf protoplasts have been made. Although about 80,000 variations in culture media compositions and in plant material have been tested, these conditions have not yet been found" (7).

Galston and his colleagues (8) have "surveyed thousands of combinations of basic nutrient media, concentrations of auxins, cytokinins, polyamines, and other growth regulating substances including coconut milk, casein hydrolysate, yeast extract and potato extract. We have combined this basic nutritional grid with variations in temperature and light treatment of the donor plant, different organs as sources of the protoplasts, osmolarity of the hypertonic medium used for protoplast isolation, nature of the osmoticum, concentration of enzyme, duration of enzyme action, and pH and temperature during cell wall digestion in protoplast isolation. Altogether, almost 20,000 variations in treatment have been employed." No sustained cell divisions, callus formation or organogenesis was obtained in these attempts with Avena sativa (oat) protoplasts.

MATERIAL AND METHODS

Protoplasts from Suspension Cultures Derived from Hypocotyls of Mature Seeds - Seeds of pearl millet (Pennisetum americanum (L.) K. Schum. var. Gahi 3, obtained from Dr. G. W. Burton, Tifton, Ga.) were germinated aseptically on 1% agar, and hypocotyls from 3-day-old seedlings were transferred to a nutrient medium (9) containing 0.25 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l naphthaleneacetic acid, and 0.01 mg/l kinetin, and incubated in the dark at 27°C. A pale white and soft callus was produced within 2 weeks. This callus was sub-cultured every 4 weeks for about 8 months. The callus was then placed in 50 ml of liquid medium in 250 ml Erlenmeyer flasks on a gyrotory shaker at 150 rpm in the dark. This provided a fast growing and highly friable suspension culture, which was subcultured every 4 days and served as the source of protoplasts. This cell line does not show any organization or differentiation into meristems, meristemoids, etc.

For the isolation of protoplasts, cells were collected from a 3-day old suspension culture on a 100 µm stainless steel filter. Approximately 500-600 mg of the freshly drained cells were added to 10 ml of a filter sterilized protoplast isolation solution (4% Cellulysin, 2% Macerozyme, 1% Driselase, 1% Rhozyme, 0.4M sucrose, prepared in the liquid nutrient medium, pH 5.8), and incubated in the dark at 14°C for 19 hr, followed by 2 hr in a shaker bath at 30°C. The protoplasts were separated from undigested cellular material by filtration through a 100 µm stainless steel filter, followed by centrifugation at 125 x g for 4 min. The protoplasts which floated to the top of the centrifuge tube were collected and washed three times with fresh medium, and cultured either in liquid droplets or mixed with an equal volume of medium containing 1.2% agar at 45°C and overlaid on agar nutrient medium in Falcon Petri dishes, with a final plating efficiency of 10^4-10^5/ml. The cultures were incubated in diffuse light in a growth chamber at 27°C, with a 16/8 hr day/night cycle. The nutrient medium used for the washing and culture of protoplasts was the same as that for suspension culture, with 0.4M sucrose and 250 mg/l glucose.

Protoplasts from Suspension Cultures Derived from Immature Embryos - Immature embryos of pearl millet, isolated 15-20 days after pollination, were grown on a medium (9) supplemented with 2.5 mg/l 2,4-D and 5% coconut milk. The resulting callus tissue was placed in liquid medium to initiate suspension cultures. The latter were sub-cultured every 5-6 days in 35 ml of the medium in 250 ml Erlenmeyer flasks on a gyrotory shaker at 150 rpm in the dark at 27°C. Protoplasts were isolated by mixing 10 ml of a 4-5 day old suspension culture with 60 ml of a filter sterilized enzyme mixture (2% Cellulysin, 1% Macerozyme, 0.5% Driselase, 0.5% Rhozyme, 0.25M sorbitol, 0.25M mannitol, 250 mg/l glucose, 3mM MES buffer, prepared in hormone-free medium at pH 5.6), incubated for 1 hr at room temperature, followed by 19 hr at 14°C in the dark. The protoplast/enzyme mixture was filtered through a layer of Miracloth, and through 100 and 50 µm stainless steel filters to remove undigested cells and other cellular debris. Protoplasts were collected and washed three times with fresh nutrient medium by low speed centrifugation (100 x g for 3 min.). At the end of each centrifugation cycle the protoplasts which were floating at the top of the nutrient medium and those which had pelleted were collected and mixed. After the final wash, the floating and pelleting protoplasts were either mixed and cultured together, or separately. Protoplasts were cultured in liquid droplets (0.25 to 0.3 ml) or in very thin layers of nutrient medium in Falcon Petri dishes (35 x 10 mm), at a density of 2×10^4 or 10^5/ml. The Petri dishes were sealed with Parafilm, incubated in diffused light in a growth chamber at 27°C, with a 16/8 hr day/night cycle. Kao and Michayluk's (10) nutrient medium was used for the washing and culture of protoplasts, without the free amino acids, nucleic acid bases, riboflavin, and vitamin B_{12}, but containing 0.4M glucose, 1250 mg/l sucrose, 2,4-D and benzylamino purine (BAP). Fresh medium with 0.2M glucose and 2% sucrose was added in drops after 15- to 20-celled colonies had been formed. Finally, fresh medium with 1% glucose and 2% sucrose was added, and after 4-5 weeks the resulting cell masses were transferred to the same medium solidified with 0.8% agar.

RESULTS

Culture of Protoplasts Obtained from Suspension Cultures Derived from Hypocotyls of Mature Seeds - Freshly isolated protoplasts are spherical but all the cellular contents are clumped together in the center of the protoplast or pushed against the plasmalemma. Within a few hours after culture, the cytoplasm becomes evenly distributed, the nucleus occupies a central position, and numerous cytoplasmic strands and streaming become visible. The protoplasts become oval to egg-shaped within 48 hr indicating the formation of a cell wall, and the first division of the newly reconstituted cell takes place after 4-5 days. Within the first week of culture, 15-20% of the protoplasts divide. Within two weeks colonies of 25-30 cells are formed. Approximately 15% of the plated protoplasts give rise to cell colonies, several of which attain a size of 1-2 mm in diameter within 4 weeks (11). These colonies are then transferred to fresh nutrient media with a lower osmoticum, and finally to a regular medium for continued callus growth. Our attempts to induce organogenesis in these protoplast-derived callus tissues have so far not been successful.

Culture of Protoplasts Obtained from Suspension Cultures Derived from Immature Embryos - The embryogenic suspension cultures are comprised of

two distinct types of cells: one large and highly vacuolated, and the other small and densely cytoplasmic. The vacuolated cells are the first to form protoplasts, which float to the surface of the enzyme solution. The vacuolated protoplasts regenerate a cell wall but were never seen to divide under the conditions of culture used in our experiments. The richly cytoplasmic and non-vacuolated protoplasts always pellet during centrifugation. These protoplasts, which are derived from embryogenic cells in suspension cultures, regenerate cell walls and form 15- to 20-celled colonies within 2 weeks after isolation and culture. The protoplast-derived cell masses are so tightly packed with small and richly cytoplasmic cells with thin walls that often it is difficult to identify the boundaries of individual cells. Further cell divisions follow in rapid succession, without significant cell enlargement, resulting in the formation of cell masses which are reminiscent of the early stages of embryogenesis (12).

The highest plating efficiency obtained by us in the culture of embryogenic protoplasts was 3-4% when 1 mg/l each of 2,4-D and BAP were added to the nutrient medium, but such cultures showed the formation of polyphenols which proved to be detrimental for further growth. No polyphenols were formed when only 2,4-D (2.5 mg/l) was used, but the plating efficiency was reduced to 1-2% (12).

Cell masses derived from embryogenic protoplasts were transferred to fresh nutrient medium for continued growth after they had attained a size of 1-2 mm. The transfer of cell masses from the liquid nutrient medium to an agar medium without any growth substances resulted in the growth of cells in tight and discrete groups, and finally led to the formation of embryoids and plantlets with shoots and roots within 4-5 weeks. The embryoids look like monocotyledonous embryos, showing a distinct scutellum enveloping an embryonal axis. The scutellar cells often proliferate resulting in the formation of secondary embryoids and plantlets (12).

DISCUSSION

Our results demonstrate that cereal protoplasts, like the protoplasts of many other herbaceous dicotyledonous species, are totipotent and embryogenic, and will give rise to plantlets under suitable conditions of isolation and culture. The fact that pearl millet protoplasts form plantlets through the process of somatic embryogenesis, and not after extensive callus growth, is of added advantage and significance in the recovery of normal plants. This first demonstration of totipotency of cereal protoplasts should encourage further vigorous efforts with other cereal species, so that somatic hybridization and genetic modification technology can be adopted for the improvement of cereal crops.

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DIVISION OF CELLS REGENERATED FROM MESOPHYLL PROTOPLASTS OF WHEAT (*TRITICUM AESTIVUM* L.)

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INTRODUCTION

Plant protoplasts offer an important biological tool for fundamental physiological and biochemical studies as well as for plant breeding (1,2). During the last decade, plants have been regenerated from isolated protoplasts of a number of plant species (2-4). Regeneration of plants from cereal protoplasts has not been successful in spite of the efforts of several laboratories. Failure to regenerate cereal protoplasts has hampered the application of protoplast technology in practical plant breeding programmes. However, recently cell clusters (even callus tissues) have been obtained from protoplasts isolated from cultured tissues of cereals (e.g. corn, rice, barley, sorghum, *Triticum monococcum* and sugar cane) (5-9). Protoplasts isolated from differentiated stem tissue of corn and rice, have also been shown to form cell walls. The division of these cells, however, resulted in the formation of non-morphogenetic cultures. Sustained division of cereal protoplasts isolated from leaf mesophyll tissue has never been observed, although mitotic division of the regenerated cells has been reported in a few cases (10-12). Therefore, it has been questioned as to whether the cereal protoplasts isolated from leaf mesophyll tissue are totipotent at all. In the present paper we report the division of cells regenerated from mesophyll protoplasts of wheat.

MATERIALS AND METHODS

Seeds of *Triticum aestivum* cv. Cheungan 1 were surface sterilized and germinated in a sand culture at 22-24°C in the dark. The first leaves of 4 to 6-day-old etiolated seedlings were used for the isolation of protoplasts.

The leaves were surface-sterilized by 70 % ethylalcohol and 0.1 % $HgCl_2$ (3 to 4 min). The lower epidermis of the leaves was removed and the tissues were submerged in a medium the composition of which is shown in Table 1. After incubation at 24°C for

3 h, the undigested material was removed by sieving through a stainless steel net. The protoplasts were collected by low speed centrifugation (3 min; 500 rpm) and washed four times (see Table 1)

TABLE 1 Composition of the medium used for protoplast isolation

	Digestion mixture[1]	Washing solution
Cellulase[2]	1.2 %	-
$CaCl_2 \cdot 2H_2O$	3.5 mM	6.0 mM
KH_2PO_4	0.7 mM	0.7 mM
Mannitol	0.5-0.6 M	0.5-0.6 M
pH	5.6	5.6

[1] Passed through a membrane filter (0.45 µ) before use

[2] Product of Tungfeng Biochemical Factory, Shanghai, China.

The protoplasts ($5x10^4$ - $1x10^5$ per ml) were incubated under sterile conditions in flat glass bottles (4 cm in diameter) in a solid or liquid medium (see Table 2). Before autoclaving, the pH of the medium was adjusted to 5.8 with 0.5 N KOH. After culturing for 6 to 12 days, one half of the cultures was transferred on the surface of the same agar medium (0.6 %) containing 0.3 M sucrose instead of another carbon source. An equal amount of liquid medium was added to the other half of the cultures which were then incubated at 25-28°C in the laboratory in diffuse daylight.

We also used Potato Medium P5 for culturing wheat leaf protoplasts. This medium contains 20 % potato extract (13), Fe-EDTA (27.8-37.3 mg/l), thiamine-HCl (1 mg/l), sucrose (15 mM), glucose (0.5 M), and agar (0.6 %) at pH 5.6. Alternatively, Potato Medium P3 was used, supplemented with 2,4-D (0.5 mg/l), 6-BA (0.5 mg/l), and mannitol (0.5 M) instead of glucose. The cultures were incubated at 20-23°C in the dark. Each experiment was repeated 5 times.

TABLE 2 Culture medium for protoplasts

Mineral salts	(mg/l)	Organic constituents	(mg/l)
NH_4NO_3	270	meso-Inositol	100
KNO_3	1480	Folic acid	0.4
$CaCl_2 \cdot 2H_2O$	900	Glycine	1.4
$MgSO_4 \cdot 7H_2O$	800	Nicotinic acid	4.0
KH_2PO_4	80	Thiamine·HCl	4.0
$FeSO_4 \cdot 7H_2O$	27.8	Pyridoxine·HCl	0.7
$Na_2 \cdot EDTA$	37.3	Biotin	0.04
H_3BO_3	2	L-arginine	50
$MnSO_4$	5	NAA	1.5
$ZnSO_4 \cdot 7H_2O$	1.5	6-BA	0.8
KI	0.025	Sucrose	0.05M
$Na_2MoO_4 \cdot 2H_2O$	0.10	Glucose	0.6-0.7 M
$CuSO_4 \cdot 5H_2O$	0.015	Xylose	500
$CoCl_2 \cdot 6H_2O$	0.01	Ribose	500
		Agar	0.6 % or without
		pH	5.8

In some experiments the culture medium was supplemented with coconut milk (5 %) and TIBA (0.5 mg/l).

RESULTS

A large number of viable protoplasts were obtained from etiolated wheat mesophyll tissue, using a single enzyme (cellulase) system (Fig. 1-1). All protoplasts contained yellowing chloroplasts. After incubation for 2 to 3 days, the protoplasts, incubated in a solid medium ($1x10^5$ protoplasts/ml), started to elongate. In 6 to 7 days, a rearrangement of chloroplasts and cytokinesis was also observed in a number of protoplasts. A few cells started to divide (Fig. 1-2). The cultures were then transferred onto a fresh solid medium containing sucrose instead of another carbon source. In about one month the development of small cell clusters was observed (Fig. 1-3).

When incubated in a liquid medium containing 0.6-0.7 M glucose, some of the protoplasts started to enlarge and to take up an oval shape. After 6 to 8 days in culture, 30 to 60 % of the surviving cells started to divide. Cells with centrally located nuclei, in general did not divide. However, there were excep-

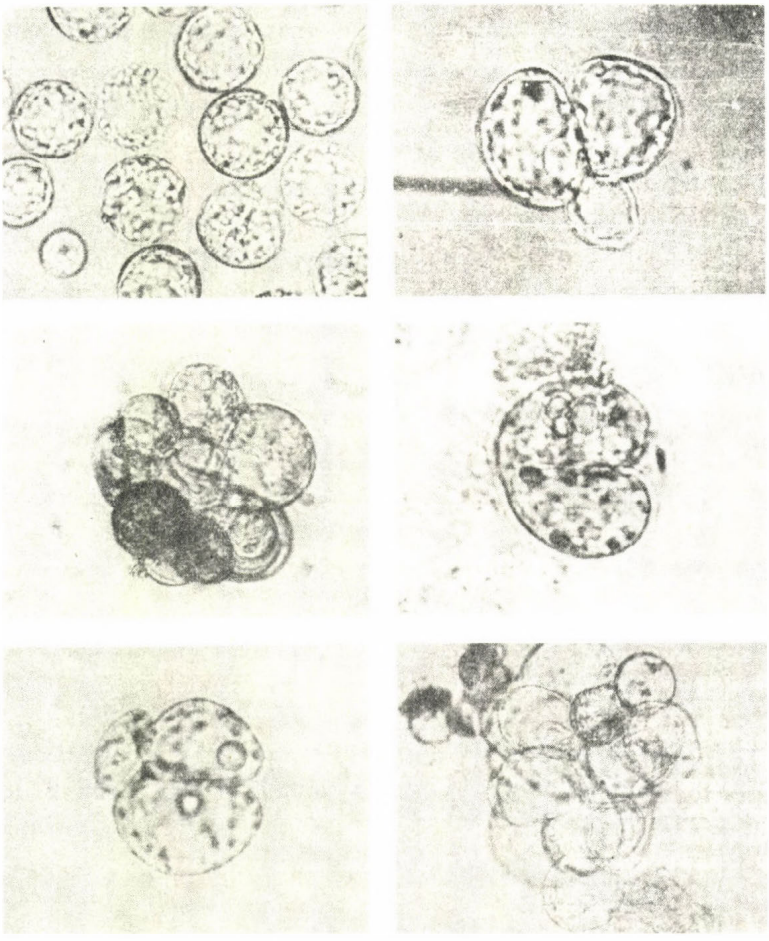

Fig. 1. Cell division in culture of protoplasts isolated from wheat leaf mesophyll tissue: 1. Yellowing protoplasts from mesophyll cells of etiolated wheat leaves. 2. First division of regenerated cells on a solid medium. 3. Cell clusters formed after culturing the cells for 15 days on a solid medium. 4. Start of cell division in a liquid medium. 5. First division of regenerated cells after culturing for 7 days in a liquid medium. 6. Cell cluster produced after transfer of the cells to the same solid medium containing, however, 0.3 M sucrose.

tions, a few of which are shown in Figs. 1-4 and 5).

When the cultures incubated for 3 days were transferred into a liquid medium containing 0.3 M sucrose, a large number of protoplasts died within 24 h. This indicates that not all protoplasts formed new cell walls during this period. When, however, the transfer took place after culturing the protoplasts for 6 to 12 days, essentially all protoplasts had already regenerated cell walls and in a few more days some cell clusters developed (Fig. 1-6).

On potato solid medium P3 and P5, the major part of the protoplasts underwent swelling, took on an oval shape, and regenerated cell walls within 3 to 4 days. The first typical cell divisions were observed after 6 to 8 days (Figs. 2-2 and 3). Some of the regenerated cells underwent a second division as well (Fig. 2-4). Bigger cell clusters, however, were not obtained.

The active ingredients of the potato media will have to be identified and the best cultural conditions established.

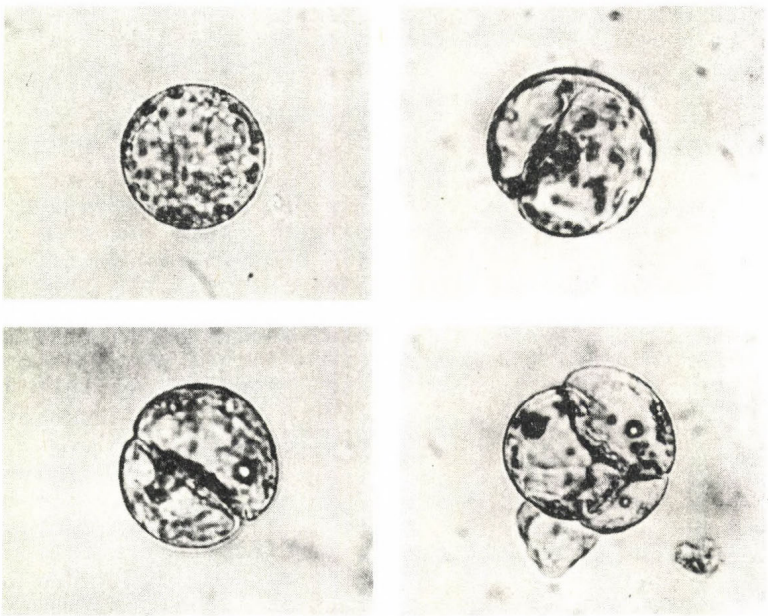

Fig. 2. Division of cells regenerated from leaf mesophyll protoplast of wheat on potato solid medium: 1. Expansion of protoplasts after culturing for 36 h. 2. Start of cell division after 4 days. 3. First division of regenerated cells after 8 days. 4. Second division of regenerated cells after 11 days in culture (Magnification 480 x).

DISCUSSION

In spite of the modest results achieved, cereal protoplasts continue to attract interest. Much attention has been devoted to the optimal conditions of culturing cereal protoplasts and a high number of data accumulated (5,6). These might prove to be useful in the future but they did not solve the problem.

In the present paper a new approach is described. We have found that budding and fragmentation of the protoplasts (sub-protoplast formation) takes place if a low osmotic medium (0.4 M) is used. The swelling of the protoplasts can be reduced by increasing the molarity of the osmoticum. Under these conditions, there is enough time for the regeneration of the cell wall and protoplast fragmentation becomes negligible. A correct balance of growth substances (synthetic auxins and phytohormones) might be crucial in this respect. The addition of coconut milk, however, does not seem to be necessary. The slowing down of swelling is beneficial for the division of regenerated cells as well. The elongation of protoplasts is not necessarily an indication of new cell wall formation. Under the conditions applied, cytokinesis was found to be a common phenomenon but it was not necessarily followed by or associated with the division of nuclei. Nuclear division appears to be governed by a number of factors including the ionic milieu, the cell cycle, and the amount of DNA synthetised. The increase in $CaCl_2$ and KNO_3 concentrations highly promoted the division of corn and sugar-cane protoplasts (6).

The optimal conditions necessary for the sustained division of cereal protoplasts have not yet been found. We were unable to obtain sustained division if the protoplasts were cultured in a thin layer of liquid medium. However, cell clusters were readily formed if the protoplasts, after 10 to 12 days in a liquid culture, were transferred to a solid medium. According to Eriksson (14) the protoplasts immersed in a liquid medium may produce ethylene and this can lead to an inhibition of cell division.

Our results indicate that wheat and barley protoplasts should be cultured, at least in the initial period, in dim light. Tobacco mesophyll protoplasts require more intense illumination. Barley protoplasts, if exposed to 1000 lux (fluorescent light), suffer serious damage and their culture medium exhibits brown discoloration. The protoplasts obtained from etiolated wheat seedlings are adapted to darkness or dim light. However, in the later period of culturing, the light intensity should be increased in this case as well.

The recent progress achieved in culturing cereal leaf protoplasts (13) suggests that leaf mesophyll cells do have a potential for regeneration.

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PROBLEMS IN PLANT REGENERATION FROM PROTOPLASTS OF IMPORTANT CROPS

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Although much of what is written in this short article may be obvious to many and can be found scattered amongst the literature we feel that the problems in regenerating plants from protoplasts are so central to our aims that it is sometimes worthwhile to remind ourselves of the barriers which we face and which must be overcome if we are to progress.

Plant regeneration and propagation are essential components of any attempted crop improvement scheme in culture (e.g. genetic modification by mutation, somatic hybridisation or nucleic acid transfer). Success in these schemes relies upon manipulation at the single cell level. Although substantial progress has been made in regenerating plants from complex explant-derived cultures of numerous nutritionally important crops there are very few cases of plant regeneration from cultures of single cell and protoplast origin. Even in cases where this has been achieved (for review see 1) other problems such as non-reproducibility and genotypic variation prevent proper application. In Brassica napus it is possible to reproducibly induce callus colony formation from mesophyll protoplasts of a spontaneous amphihaploid strain (2). However, of the thousands of calluses produced from the protoplasts, less than 10 ever formed shoots or embryos. Further this regeneration was spontaneous and could not be controlled by alterations in the culture medium. In part the problem may be overcome through the use of protoplasts from explants other than leaves such as propagating stem embryogenic cultures of rapeseed (H.W. Kohlenbach, personal communication). Similarly, in cereals which are proving extremely recalcitrant to protoplast technology, it may prove worthwhile to attempt to isolate protoplasts from organs or cultures which are known to possess cells capable of forming plants e.g. immature embryos of maize (3) and sorghum (4), cultured roots of rye (5), developing inflorescences of wheat (6), maize (P. Gordon, personal communication) and sorghum (Brettell and Thomas, in preparation) or cultures derived from these sources. The potential of this latter approach is clearly

shown by the very recent results of Vasil and Vasil (personal communication). It was possible to isolate protoplasts from suspension cultures of Pennisetum americanum and to regenerate plants via embryogenesis from the protoplasts. The suspensions were derived from cultures of immature embryo origin. Other obvious ways to attempt to solve current problems include (i) improving protoplast isolation techniques including enzyme technology, (ii) screening of different media to determine the conditions inducive or permissive of expression of morphogenetic potential, (iii) searching for new endogenous or synthetic phytohormones or anti-phytohormones and (iv) altering the environment under which donor plants are grown including the use of sterile shoot cultures and the preculture of plant organs before protoplast isolation. The use of many of these approaches may require the development of techniques for handling smaller numbers of protoplasts than would be available from mesophyll tissue and for purifying populations of protoplasts in which the individual units may be of widely different buoyant density. Examples of such techniques are the microdrop array technique (7), the multidish (8) and density centrifugation (8,9).

Why do only very few of the protoplast-derived colonies of haploid rapeseed give rise to plants? Why do mesophyll protoplasts of several legumes form callus which fail to form plants? Why do mesophyll protoplasts of cereals refuse even to divide? At this stage in development of the field there is certainly little evidence to suggest that all living cells are totipotent. In 1969 Halperin (10) raised the intriguing question of whether structures formed in plant tissue cultures may originate only from a limited number of cell types which are present in the plant and which are carried over into cell culture during callus initiation. One could extend this view and suggest that although in a particular tissue all cells appear morphologically alike there may be only some cells which are genetically or physiologically able to form plants (the term genetically includes both nuclear and cytoplasmic genetic material). Some cells in the intact plant may be incapable of division even upon isolation. Other cells though capable of division and thus of forming a cell culture, may retain certain differentiated functions which restrict their morphogenetic potential. Similar concepts are invoked by postulating that during the course of evolution certain cells in certain tissues of certain species have become so highly specialised that it may never be possible to obtain plants from the cells. Alternatively, all living cells may be potentially able to form a complete new organism but we do not yet know how to permit this property to express itself. In reality the problems we face are probably a result of interactions of all the above-mentioned phenomena.

Most workers in this field tend to permit the formation of a large callus colony before attempting to obtain shoots. Further from protoplast to callus colony the phytohormone content of culture media is seldom changed. However, our recent evidence with protoplasts of Hyoscyamus muticus and Nicotiana tabacum (Wernicke and Thomas, in preparation) suggests that this may lead to a selection of non-morphogenetic cells at the expense of morphogenetic ones. The complexity of the tissue culture situation is depicted diagrammatically in Fig. 1. It has long been postulated that the initiation of morphogenesis occurs only once in any particular culture, that is during culture isolation. The result is the formation of "proembryogenic masses" (11) or "organized aggregates" (12). The continued presence of high phytohormone levels in the culture medium suppresses the full expression of morphogenesis but causes a proliferation of the organized structures formed during culture initiation. Provided that organized structures are present in the culture these will give plantlets (or roots!!) when the external auxin supply is lowered either by transference to auxin-free medium or by a natural depletion caused by culture growth. However, during the period of morphogenetic suppression by phytohormones there can occur the formation and preferential selection of rapidly growing cell types apparently lacking morphogenetic ability and if these latter cells proliferate faster than the organized structures an apparent loss of morphogenetic ability will occur. One could extrapolate this concept and propose that the formation and preferential selection of cell types apparently lacking morphogenetic potential can occur directly from the initial explant or the initial protoplast population. Such a phenomenon could easily explain the failure to observe shoot formation from leaf protoplast calluses of legumes and the failure to observe shoot formation from most of the protoplast-derived calluses of rapeseed. Our observations with H. muticus and N. tabacum suggest that it is absolutely essential to attempt to obtain plantlets from cultures by adjusting the phytohormone levels at as early a stage as possible. If this cannot be achieved in all likelihood one can never obtain morphogenesis in the cultures.

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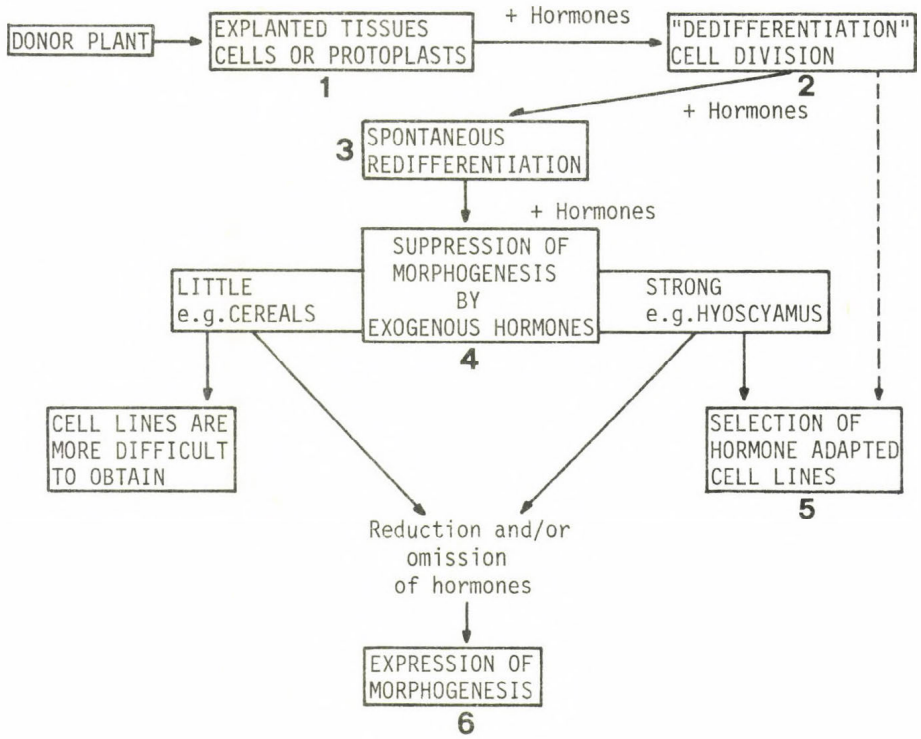

Fig. 1

1 - The isolation procedure (isolation or wound shock) makes the cells competent to "dedifferentiate" and

2 - to divide under the influence of high hormone levels.

3 - After the first divisions redifferentiation occurs spontaneously giving rise to primordia requiring different levels of hormones.

4 - When leaving the cells under high hormone levels full expression of morphogenesis is inhibited but proliferation of the primordia occurs.

5 - In cases where this inhibition is very strong fast growing cell variants adapted to the high hormone levels may be eventually selected. The whole culture will lose its morphogenetic potential if the variants are non morphogenic.

6 - Reduction and finally omission of hormones lead to full expression of morphogenesis (embryos, shoots or roots) determined by endogeneous hormones.

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ADVANCES IN PLANT PROTOPLAST RESEARCH IN CHINA

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INTRODUCTION

Ever since the potentiality of using plant protoplast cultures in crop improvement was pointed out at the International Conference on Plant and Tissue Culture for Crop Improvement, held in Italy in 1969 (1), much effort has been made to regenerate protoplasts into intact plants, to demonstrate the uptake of foreign genetic material by protoplasts and to obtain somatic hybrids by protoplast fusion (2). The techniques applied for the isolation, culture and fusion of protoplasts, as well as those employed for the selection of fusion products, developed rapidly (3). It became evident that protoplasts provide an excellent system for the study of somatic cell genetics, genetic recombination, and genetic modification. In addition, protoplasts were found to be useful in studies on the structure and function of plant cell membranes and in plant virology. However, it is a long way from academic research to practical application. Protoplast research in China was initiated in 1973 (4). Techniques have been developed for the preparation, culture, and genetic manipulation of protoplasts from cereal plants, the Leguminosae and Solanaceae (5,6). This paper summarizes the results of recent work done in this field in China.

Isolation of Protoplasts

Although protoplasts have been isolated from various tissues of a number of plant species, not all plants yield viable protoplasts in high numbers. Difficulties encountered in the culture and regeneration of protoplasts are even more common. A number of factors affect the production and viability of protoplasts, such as (a) the age of the plant, light regime, developmental stage, photoperiodic conditions, temperature, humidity; (b) the composition of the enzyme solutions, concentration of osmotic stabilizers; (c) the duration of protoplast isolation, temperature, isolation techniques etc. In this respect, there are great differences among the various plants and tissue. In China, protoplasts are isolated both from mesophyll cells and callus tissues.

The cereal plants, from which protoplasts have been isolated, are listed in Table 1.

TABLE 1 Isolated protoplasts of cereal crops

Plant	Cell origin	Yield of protoplasts per ml	Reference
Rice	Haploid callus	$1x10^6$ - $3.2x10^6$	7
Rice	Shoot tip	$1.35x10^5$ - $2.4x10^5$	8
Wheat	Young leaf	$1x10^5$ - $4x10^5$	5
Barley	Young leaf	$5x10^5$ - $1x10^6$	6
Corn	Young leaf	large number	9
Rye	Young leaf	approach all	10
Avena	Young leaf	large number	11
Corn	Root tip	$1x10^4$	12

Ecological conditions. In the wide majority of cases, protoplasts were isolated from the leaves of young (5 to 10-day-old) seedlings. The temperature and the light regime often play an important role. For example, the protoplasts isolated from the leaves of barley seedlings grown at 28°C under long-day conditions were found to have large vacuoles and a very fragile plasma membrane. In contrast, the protoplasts obtained from plants grown at 20°C, under a short-day light regime, were more suitable for culturing and were obtained in a much higher yield (6).

The ecological conditions greatly affected the yield and quality of protoplasts obtained from wheat leaves. Recently, protoplasts have been isolated from 3 to 6-day-old, etiolated wheat seedlings, grown in the dark at 25°C. These protoplasts were shown to divide in culture. The yellow colour of the chloroplasts was found to be an excellent marker in the identification of fusion bodies when such protoplasts were used in fusion experiments. The reproducibility of experiments carried out with protoplasts from etiolated wheat seedlings was high.

Tissue sources. Since, in some cases, it is difficult to isolate protoplasts from mesophyll tissues, and it is especially difficult to regenerate plants from them, cultured callus tissues were also used as a source of protoplasts.

Chi-kuei Tsai et al. (7) subcultured rice calli 7-8 times on LB medium and obtained vigorously-growing tissues. Good quality protoplasts were obtained from 8-day-old suspension cultures of rice cells derived from these calli. Protoplasts isolated from the cul-

tured cells regenerated the cell wall and divided readily. However, it was found to be difficult to induce differentiation in these calli because of the previous long subculturing. Therefore, in the Institute of Botany, Guandong Province, protoplasts were prepared from the stem tip of hybrid rice seedlings, by the use of incubation mixtures containing cellulase, pectinase, and mannitol. 1.35 - 2.4x10^5 spherical protoplasts were obtained per gram of fresh tissue material. As also shown by Potrykus for maize, in these types of protoplast cultures the induction of differentiation is more effective than in those derived from callus tissue cultured for a longer period.

Enzyme solutions. Most laboratories in China used cellulases prepared by the laboratories themselves (14, 17). These preparations are actually mixtures of enzymes. Protoplasts isolated from petunia (15), tobacco (18), and carrot (16), by using these enzyme preparations, were regenerated into entire plants. In this respect, the preparations gave identical results with those obtained with Onozuka R-10 made in Japan. Recently, the enzyme was extracted from *Trichoderma viride* AE_3-867 by the Factory of Biochemical Reagents at the Institute of Biochemistry, Shanghai. The preparation is now being produced on a commercial scale.

2. Protoplast Culture

Plants have been regenerated, in China, from isolated protoplasts of tobacco, petunia, and carrot in several laboratories (15, 18-21). Much work has also been done on protoplast cultures of cereals and legumes. The results obtained are summarized in Table 2.

Culture methods. A solid medium was used by Wen-an Li et al. (18) to culture tobacco mesophyll protoplasts. After 8 to 10 days of culture in modified NT medium, cells which regenerated new cell walls were transferred, together with a piece of agar attached to them, to the same solid medium containing identical or lowered amounts of osmotic stabilizer, respectively. In contrast to the non-transplanted cells, which did not divide even in 30 to 40 days, the transplanted cells underwent comparatively rapid cell divisions and formed calli. Protoplasts obtained from haploid plants divided more slowly than those isolated from diploid plant material. Since it was not very convenient to work with the solid medium, a two-layer method has recently been introduced (the upper layer liquid, the lower layer solid). By the use of this method, whole plants have been regenerated from isolated protoplasts of *Nicotiana rustica* and *N. alata* (31).

In our experiments, viable cell clusters did not develop if the protoplasts were kept in the liquid medium (18). We believe that the use of a solid culture medium has proved and will prove to be useful in the culture of barley (22), wheat (33), and sorghum (25) protoplasts. According to Eriksson et al. (23,24), protoplasts submerged in a liquid medium may readily form ethylene. The latter might prevent DNA synthesis and cell division. This aspect of the problem is worth studying especially with

those species the protoplasts of which are difficult to culture.

TABLE 2 Plant protoplasts cultured to form dividing cells and plants in China

Plant	Source of protoplasts	Regenerated products	Culture methods	Reference
Nicotiana tabacum L.	leaves	plants	liquid	20
	callus		solid	18
			liquid	19
Nicotiana glauca L.	leaves	plants	liquid	21
Nicotiana rustica L.	leaves	plants	liquid	a
Nicotiana alata L.	leaves	plants	liquid	b
Petunia hybrida L.	leaves	plants	liquid	15
Oryza sativa L.	haploid callus	callus	liquid	23
Triticum aestivum L.	leaves	cells	solid	26
Hordeum vulgare L.	leaves	cells	solid	22
Secale cereale L.	leaves	cytokinesis	liquid	10
Vicia faba L.	leaves	cells	liquid	c
Daucus carota L.	root plants	plants	liquid	d
Daucus carota L.	callus	plants	liquid	16
Ipomea batatas Lam.	root	callus	liquid	f
Jerusalem artichoke	root callus	cells	liquid	e

a, b, d Hsia Chen-au et al.

c Sun Yong-ru et al.

e Lin Zun-pin, Ma Chen et al.

f Wu Yao-wu et al.

Tissue sources. Although protoplasts can be isolated from practically every organ of a plant, most plants listed in Table 2 have been regenerated from leaf mesophyll protoplasts or those obtained from callus tissue (tobacco, carrot). In tobacco, there was no major difference between these two tissue sources, except that the protoplasts obtained from callus tissue divided faster than those obtained from leaf mesophyll. The division frequency of the former was 70 %, that of the latter 45.9 % (18).

Protoplasts isolated from leaf callus have shown a comparatively low regeneration capacity. In particular, it was difficult to induce root formation from such calli. In contrast, the protoplasts isolated from freshly induced stem callus were endowed with a high regeneration potential.

Plants have not yet been regenerated from the mesophyll or callus protoplasts of cereal plants (2,3,29). Preliminary results suggest that the use of protoplasts from callus tissues or from stem meristems is more promising. Still, the redifferentiation of protoplasts obtained even from callus tissues seems to be difficult at present. Division of barley mesophyll protoplasts has been observed in our laboratory. However, the frequency of division was low (0.1 %). It is apparent that the media and the methods applied have to be improved.

Culture medium. Chi-quei Tsai et al. (19) compared the effects of NH_4NO_3 and $(NH_4)_2SO_4$ on the development of protoplasts obtained from rice callus. NH_4NO_3 inhibited the growth of calli and in its presence loose callus tissues were obtained. A high number of small calli developed, if the cultures were shaken. In shaken cultures, the addition of $(NH_4)_2SO_4$ to the medium stimulated the growth of calli. The addition of vitamins and other organic compounds also proved to be beneficial. In NT medium, the tobacco mesophyll protoplasts divided much faster if 250 mg per liter of lactalbumin hydrolysate was added to the medium. The division frequency was also raised from 50 % to 80 % under these conditions.

Carbon source and osmoticum. In our work with wheat protoplasts, glucose and some other sugars (if supplied in a lower concentration) proved to be the best carbon sources (33). Budding and anuclear subprotoplast formation was common if a low osmotic medium (0.4 M glucose) was used. These phenomena are associated with the rapid swelling of the protoplasts. During the spring and the summer, the increase of molarity of glucose to 0.6-0.7 M greatly reduced these disadvantageous phenomena.

It is important to stress that typical division of cells regenerated from mesophyll protoplasts of barley (22) and wheat (33) were obtained on a simple potato medium containing 20 % potato extract and a few other compounds.

Auxins and other phytohormones. In our experiments with *Nicotiana rustica*, different plant types were regenerated, depending on the phytohormone used. On some of the calli flower buds developed which flowered and set seeds. This type of system might be extremely useful in studies on the differentiation in plants.

Some other laboratories are working on protoplasts isolated from other species. Several divisions of the cells regenerated from the protoplasts obtained from *Jerusalem artichoke* callus tissue were observed on a modified MS medium. Calli were obtained from the mesophyll protoplasts of sweet potato (*Ipomea batatas*) in a liquid medium (34).

3. The Genetic Modification of Protoplasts

The protoplast system proved to be useful in the genetic modification of somatic cells by increasing the frequency of genetic recombination and the rate of mutation (27).

Fusion products were obtained in our laboratory from homologous partners by fusing mesophyll protoplasts of wheat, maize and some other plants. Heterologous fusion products of wheat and *Vicia faba* protoplasts have also been obtained (12). In these experiments $NaNO_3$ was used as a fusogenic agent and a fusion frequency of 40 % was found. Later, using Kao's fusion technique (PEG), and high pH and Ca^{++}, interspecific fusion products were also obtained by fusing protoplasts from etiolated wheat and green petunia leaves (25 % fusion frequency was obtained). 10 % of the fusion bodies represented heterogeneous multi-protoplast structures. Nuclear staining revealed that the fusion bodies were heterokaryons. Some of the fusion products, transferred to modified Ds2 medium, regenerated cell walls and underwent cell division. On a fresh medium, more than 20 calli were obtained. The hybrid nature of the calli could not be ascertained, since a suitable selection system, specific for the hybrids, was not at our disposal.

Chloroplast transplantation experiments have also been carried out in China. By the use of PEG as a fusogenic agent, wheat and spinach chloroplasts were introduced into carrot protoplasts isolated from callus tissue. 2-5 % of the protoplasts took up foreign chloroplasts. These experiments are in progress.

Tian Bo et al. (28) investigated the infection of tobacco (*Nicotiana tabacum* cv. Samsun) mesophyll protoplasts with rape mosaic virus (YMV_{15}). YMV_{15} is serologically related to TMV but it also infects rape and Chinese cabbage. In the presence of polyornithine 46-94 % of the protoplasts were infected. Each infected protoplast contained $1\text{-}3 \times 10^5$ virus particles. In addition to the virus work, these workers exposed cultured tobacco cells to the mutagenic agent EMS and cultured the cells under the selective pressure of added 8-azaguanine and 2-thiouracil. Resistant calli were isolated and shown to maintain this property through several generations.

Research work on protoplasts started in China rather late. Consequently, several problems will have to be solved in the future, e.g.: (a) regeneration of plants from the protoplasts of cereal plants, legumes, and other important crops; (b) the provision of plant material with known genetic background and selective markers; (c) study of the possibilities of obtaining hybrids of distantly related plants. These programmes require more knowledge on cell differentiation and genetic stability. Investigations along these lines are in progress.

In addition to the protoplast work, some laboratories are developing tissue culture systems (for maintenance of stock material). Such cultures have already been established for rice, wheat, peanut, sugar-cane, carrot, and artichoke.

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PROTOPLAST FUSION, MECHANISM AND CONSEQUENCES FOR POTATO BREEDING AND PRODUCTION OF POTATOES + TOMATOES

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A. Some Experiments Aimed at Understanding the Surface Structure of Protoplasts

Grout et al. (1973) and Nagata and Melchers (1978) have observed the negative surface charge of freshly prepared protoplasts, in electrophoresis experiments. Ca^{++} ions reduce the negative surface charge more and more with increasing concentration. ζ-potential = 0 mV and coagulation is attained with a 100 mM concentration of $CaCl_2$. This result makes the effect of $CaCl_2$ and high pH in producing protoplast fusions more understandable (Keller and Melchers, 1973). The polycations, polylysine and polyornithine, increase the surface charge from -30 mV ζ-potential to +30 mV, but the protoplasts do not survive such changes. After resolution of the polycations in short-term experiments, the negative surface charge returns. Histones destroy the protoplastic membranes after reducing the charge to zero. In preliminary experiments, the influence of some enzymes on surface charges was studied. α-Neuraminidase is, through removing the sialic acid from the surface, active in reducing the negative charges of animal cells. However, in plant protoplasts, α-neuraminidase has no effect. Phospholipase C only destroys the surface, but acid phosphatase reduces the negative charges considerably (40-50 %). After resolution of the enzyme, the reduction in negative charges, our rough draft of the plant protoplast surface is momentary: negatively charged phosphate side-chains are partially covered by proteins.

Since Okada (1962), fusion of animal cells has been induced mainly by inactivated Sendai virus. The active part of this fusion agent seems to be the phospholipid of the virus membrane (Hosaka and Shimizu, 1972; Lucy, 1970). Plant protoplast experiments with natural phospholipids like lysolecithin did not lead to definite results (Potrykus and Hoffmann, 1973). After we became interested in changing the surface charge of protoplasts to induce an artificial sexuality (Melchers, 1977), H. Eibl entered into cooperation with us (Nagata and Melchers, 1978; Nagata et al., 1979) whereby he synthesized completely artificial phospholipids (Eibl, 1978; Eibl and Nicksch, 1978). 1,2-O-dipentadecyl-methy-

lidene-glycerol-3-phosphoryl-(N-ethylamino)-ethanolamine (Fig. 1) produces protoplast fusions to a lesser extent than PEG and PVA (Nagata, 1978), but very quickly, in contrast to the well known agents. This may become important when we want to fuse many protoplasts in a double layer produced by artificially positively and naturally negatively charged protoplasts (Melchers, 1977). Ca^{++} ions are most necessary in the fusion solution, and this solution should be purged of large vesicles by filtering after sonification. In elucidating the molecular basis of the fusion process it may be helpful to use simple phospholipids with different exactly known structures rather than mixtures of natural products.

B. Fusion Hybrids in Potato Breeding

It seems to be extremely necessary not only to play "l'art pour l'art" with our protoplast work and other unconventional methods of plant physiology, genetics and breeding, but to use these in combination with well-known conventional methods. Wenzel et al. (1979) propose an "analytical-synthetic" way of combining production of dihaploid potatos by inducing parthenogenesis through crossing the cultivars with *Solanum phureja*. Selection for the useful characters in the dihaploid state and eventual crossing of two such lines follows. If one wants to go ahead to a tetraploid line while maintaining the heterozygosity *and* uniformity of two dihaploid lines, the best way to do this is to fuse protoplasts. During this meeting Wenzel will give a more detailed report and explain what is going on at Köln-Vogelsang.

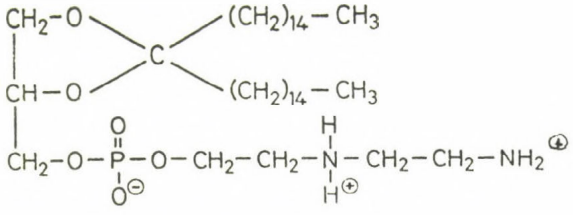

Fig. 1 1,2-O-Dipentadecylmethylidene-glycerol-3-phosphoryl-(N-ethylamino)-ethanolamine

C. Somatic Hybrid Plant of Potato and Tomato Regenerated from Fused Protoplasts

Not long ago we were forced to confess that cell hybrids after fusion of protoplasts are possible between very distantly related plants, but regeneration of hybrid plants is known only in cases in which sexual hybrids are also possible. Since 1978 some exceptions have been found, e.g. the potato + tomato hybrids (Melchers et al.,1978). Fusion hybrids between plants which can also be crossed sexually can be of great value (see the last chapter and Wenzel et al., 1979 for breeding purposes, or Gleba, 1979, Belliard et al., 1978; or Chupeau et al., 1978 for genetic analysis of mendelian and non-mendelian factors). People criticising the first fusion experiments between closely related plants crossable by sexual methods as "unnecessary" have been on the wrong track.

The first potato + tomato hybrids were started in 1977 by fusion of mesophyll protoplasts of tomato (*Lycopersicon esculentum* Mill. var. cerasiforme (Dunal) Alef, mutant yellow green 6, Rick) and protoplasts of a submersed callus culture of the potato dihaploid line HH 258 which were given to me by Prof. Straub of Köln-Vogelsang. Under the conditions that we used the tomatoes regenerated from callus had only roots and no shoots. At first it was not easy to decide from among the regenerating green shoots what could be a hybrid and what could be a potato with perhaps an abnormal chlorosome set. An analysis of the chromosome numbers (Dr. M.D. Sacristan, Institut für Angewandte Genetik, Berlin-Dahlem) gave some indications. The chromosomes of the potato and tomato are not sufficiently different morphologicallyto enable a strict proof by karyological analysis.

Fortunately, Dr. Holder (Carlsberg Institute, Copenhagen) found that potatos and tomatoes can be differentiated through the SDS electrophoresis of the ribulose-1,5-biphosphate carboxylase ("Fraction 1 protein"). Using this method four hybrid plants have been identified - three plants with the plastome of a tomato, and one plant with the plastome of a potato. Here I propose for the first time that we call potato + tomato hybrids with a potato plastome "pomatoes" (in German "Karmaten") and hybrids with a tomato plastome "topatoes" (in German "Tomoffeln").

In the mean time,as Professor D.V. Wettstein wrote to me, our colleagues at the Carlsberg Institute have analysed some more plants. We now have nine plants analysed - four topatoes and five pomatoes. In some of these plants Dr. Herrmann (Botanisches Institut, Düsseldorf) has studied the plastid-DNA using restriction enzyme analysis and has found no differences between this and the fraction 1-analysis of the large subunit. More details, and pictures of some topatoes and pomatoes, will be published elsewhere. Some plants are more or less monstrous and grow slowly, and some have chromosome numbers near the ideal number of the amphidiploid (48). In some plants we have found tuber-like stolons, but as yet no fertile flowers.

In former times potatos and tomatoes belonged to the same genus *Solanum*. Recently tomatoes were put in the genus *Lycopersicon*. I hope the modern systematicians appreciate our findings !

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REGENERATION AND FUSION OF PROTOPLASTS FROM IMPORTANT CROP PLANTS OF THE BRASSICEAE

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INTRODUCTION

Some of the plants belonging to the Cruciferae, especially members of the genus Brassica, are of great economic importance. For example, the vegetable kale or cabbage (Brassica oleracea) as well as oil seed plants like rape (B. napus) are important crop plants. Rape is the most productive oil plant of the northern temperate zone. Of further interest is that the Cruciferae present one of the rare examples in the plant kingdom where intergeneric sexual hybrids have been made. B. juncea (Indian mustard), B. carinata (Abyssian mustard) and B. napus were found to be interspecific hybrids, which are amphidiploids bearing genomes AB, BC and AC, respectively. B. campestris (turnip) is considered to have contributed genome A, B. nigra (black mustard) genome B, and B. oleracea genome C. The scheme, given in figure 1, often referred to as the triangle of U (1), illustrates the basic character of the diploid species at the triangle's points. One prerequisite for successful breeding of the corresponding amphidiploids is the establishment of a broad genetic basis. Because of low genetic diversity, hybrid lines of B. napus show relatively small heterotic effects (2). Therefore, synthetic amphidiploids represent a promising challenge for breeders. In addition to our protoplast work with B. napus (3), we included B. campestris, B. oleracea and B. nigra into our protoplast program and used these species in experiments on interspecific and intergeneric somatic hybridization.

RESULTS

Protoplast regeneration.

Isolated protoplasts from all four species could be regenerated into calli. In experiments with amphidiploid and amphihaploid B. napus, this was possible with greenhouse-grown

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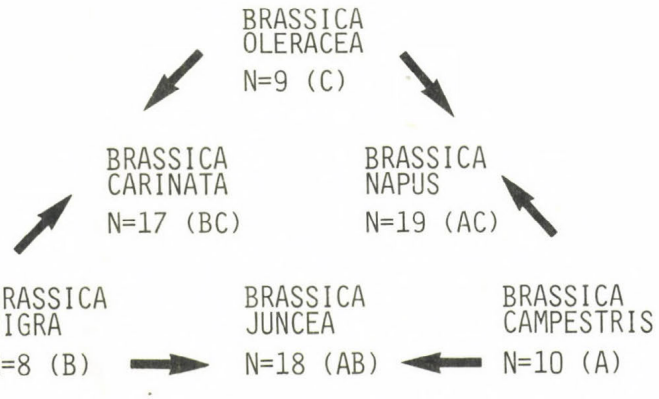

Fig. 1. The triangle of U (1), showing the origin of alloploid species of Brassica (n = haploid chromosome number).

plant material on different conventional culture media (3). In many cases, a release of brown particles could be observed in dividing cells. Figure 2 shows such "spitting cells" and a colony completely covered by these brown spots. But, in B. napus, the calli are able to outgrow these particles without special treatment and develop further. Cells of the three other species were not able to survive under the same conditions. About ten days after protoplast isolation, the brown colonies died. To obtain calli the following procedures were developed (4):

1.) Plant selection. Only sterile-grown plant material was used for protoplast isolation. From these plants, only those few were taken which had not turned the agar brown within 8 weeks after germination. These plants showed a comparatively low poly-phenol oxidase (PPO) activity as well as a reduced number of PPO isoenzyme bands in leaves, roots and agar.

2.) Culture conditions. Repetitive washing of the cultures, up to two times per week, has been shown to be neccessary. The intervals can be prolonged by lowering the protoplast titer.

3.) Control of growth rate. Only actively dividing cells release the brown particles en masse. Slow growth, combined with a reduced browning, could be attained by using a new culture medium, having no macrominerals other than 2 g/l KNO_3 (4). The addition of Ca++ led to an especially explosive release of particles. The addition of antibiotics can also reduce the growth rate.

Any combination of the procedures mentioned above may also

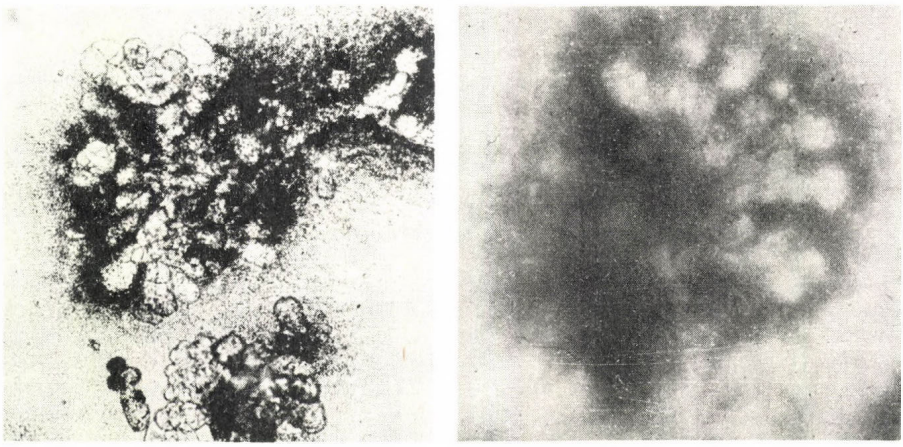

Fig. 2. a) Small cell colony developed from a isolated protoplast of B. napus showing the release of brown particles.
b) Small callus growing through the cover of brown particles.

help with similar problems in other plant species. In all experiments with B. campestris, B. oleracea and B. nigra, selection of plants (procedure 1) was neccessary. B. oleracea only survived in the minimal medium.

An explanation for these difficulties in culturing Brassica (and other) protoplasts can only be speculative (5). Some sort of infection persisting inside the plant cells or associated with them in culture can hardly be excluded. The high PPO activity level could be an indication of this (6). It may also be possible that infection within the seed was not completely eradicated by the sterilization procedure. Another explanation could be a release by the cells themselves of some toxic substances that cause the death of the colonies.

Morphogenesis.

No organogenesis could be observed in B. nigra. Roots routinely developed from calli of the three other species. Shoots and flowering plants could only be regenerated from B. napus (3). Plant formation occurred very rarely and completely without control by the experimentor. Very recently we were able to partially overcome this problem. Using protoplasts isolated from stem embryogenic tissue (3), which has developed either from cultured anthers of rape (7, 8) or from protoplast derived calli, embryogenic clumps can be obtained when 8 week old calli are transferred to a potato extract medium (9, as modified in 10). A repeated transfer after an-

other 4 weeks to a modified MS-medium (35 g/l sucrose, 2 g/l galactose, 800 mg/l glutamine, 0,5 mg/l IAA, 0,05 mg/l BAP) results in a renewed induction of stem embryogenesis and plant production. Using this procedure we are able to induce between three and six embryogenic strains per experiment. Of course, the efficiency of this induction must be increased before rape may be bred with microbiological methods (11). Nevertheless, a clear sequence of steps gives a small, but reproducible success.

Protoplast fusion.

Protoplasts of all four species were used in fusion experiments in interspecific and intergeneric combinations. In most cases, callus protoplasts from one species and mesophyll protoplasts from another were combined and visible fusion bodies were mechanically isolated and individually cultured in microdroplets (12). First divisions occurred in nine combinations (table 1), but in all but one case the colonies died before transfer to solid media. Heterokaryons of Arabidopsis thaliana + B. campestris gave rise to symmetric and asymmetric Arabidobrassica hybrid plants (13), which is a topic of another paper of this meeting. Protoplasts isolated from Arabidobrassica cell line ab 2 (14), which showed no specific chromosome elimination, were fused with mesophyll protoplasts from different species. First divisions were observed in these cells with three genomes combined by somatic hybridization.

In another type of experiment, mesophyll protoplasts of B. oleracea and B. campestris were fused in attempts to resynthesize B. napus. A presumed hybrid callus was selected on the basis of heterotic growth. Vigorous growth of hybrid cells seems to be a quite common phenomenon (14, 15) and seems to be the most suitable selection procedure. Isoenzyme analysis of esterases and amylases support the efficacy of the selection and suggest the formation of a parasexual rape callus. No plant regeneration could be obtained from the presumed hybrid cells.

CONCLUDING REMARKS

The usefulness of Brassica protoplasts in mutation breeding programs, in vitro selection or somatic hybridization is still limited due to our inability to induce controlled morphogenesis. Especially when we select for a special rare genotype or newcombination, a perfect morphogenetic capacity of the system is required. Such a system is the stem embryogenic tissue of B. napus (3), which we already use in this kind of experiment (16, 17) and which has been also useful for isolating protoplasts. We would like to suggest the stimulation of efforts on controlled morphogenesis of cultured plant cells. We believe that there is some discrepancy between the optimism caused by lists of regenerated plant spe-

cies and the reality of controlling this process.

TABLE 1 Results from Fusion Experiments with Brassica Species in Interspecific and Intergeneric Combinations

protoplasts used		development obtained from hybrid cells		
callus protoplasts	mesophyll protoplasts	divisions	callus	plants
Arabidopsis thaliana	*B. campestris*	+	+	+
	B. napus	+	-	-
	B. nigra	+	-	-
	B. oleracea	-	-	-
A. thaliana + *B. campestris* (Arabidobrassica)	*A. thaliana*	-	-	-
	B. nigra	+	-	-
	N. debneyi	+	-	-
	N. silvestris	+	-	-
	N. tabacum	+	-	-
Brassica campestris	*A. thaliana*	-	-	-
	B. oleracea	+	-	-
	N. tabacum	-	-	-
Daucus carota	*B. campestris*	-	-	-
	B. oleracea	-	-	-
Nicotiana tabacum	*B. campestris*	+	-	-
	B. napus	-	-	-
	B. nigra	-	-	-
	B. oleracea	-	-	-
mesophyll protoplasts	mesophyll protoplasts			
B. campestris	*B. oleracea*	+	+	-

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SOMATIC HYBRIDIZATION OF *SOLANUM TUBEROSUM L.* AND *SOLANUM CHACOENSE* BITT. BY PROTOPLAST FUSION

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Interspecific hybridisation of somatic cells has resulted, in certain cases, in the production of hybrid (1-5) or cybrid (6) plants. The somatic hybrids were, in most cases, similar to the corresponding amphidiploids obtained by sexual crossing. In several instances, however, the chromosome number of the somatic hybrids was found to be different (1-3).

The aim of the present work was to obtain somatic hybrids between cultivated and wild potato species. *Solanum tuberosum* L. cv. Priekulsky rannii (2n=4x=48) and *S. shacoense* Bitt. (2n=2x=24) were chosen for the experiments.

The techniques used for the isolation of protoplasts from leaf mesophyll tissue of plantlets originating from meristem cultures of both potato species have been described in previous publications (7-9). The isolation of protoplasts from callus cultures of *S.tuberosum*, and the conditions necessary for plant regeneration, have also been reported (7-9). Details of the procedure used for the isolation of protoplasts are described in Table 1.

Protoplasts isolated from callus cultures of *S.tuberosum* (8th transfer) (Fig. 1d) and leaf mesophyll protoplasts of *S. chacoense* (Fig. 1c) were mixed in 0.16 M $Ca(NO_3)_2$ in a ratio of 1:1 in a final density of 10^6 protoplast per ml. Protoplast fusion was induced by 42% (w/v) polyethylene glycol (MW 1540) according to a modified version of the Kao-Michayluk procedure (10). After 15 to 20 min. the protoplasts were washed with 0.16 M $Ca(NO_3)_2$. The washing was repeated twice with a modified Murashige-Skoog (MS) medium (11). The protoplasts were cultured in a solid Murashige-Skoog medium containing the following ingredients: macro- and micronutrients and chelated iron as in MS, vitamin B_1 (2.0 mg/l), vitamin B_6 (0.8 mg/l), 2,4-D (1.0 mg/l), α-naphtylacetic acid (1.0 mg/l), 6-benzylaminopurine (1.0 mg/l), sucrose (25 g/l),mannitol (0.32 M), agar (6 g/l), at pH 5.5-5.6. The protoplasts were cultured in

TABLE 1. Conditions for the isolation of potato protoplasts (pH 6,1, shaker 72 rpm/min, t=26° C)

Plant	Chromosome number	Number of viable protoplasts	Isolation conditions		
			Enzymes	Concentration of mannitol (M)	Time of incubation with enzymes (hours)
	2n=4x=48	7.10^6 (90% viable)	2% xylanase	0.4	6
S. tuberosum					
	2n=2x=24	$2,5.10^6$ (80-85% viable)	2% xylanase+ +0,5 Onozuka R-10	0.4	7-8
	callus tissue	2.10^5 30% cells	3,5% xylanase	0.4	9
S. chacoense					
	2n=2x=24	$5,6.10^6$ (70% viable)	2% xylanase	0.3	5

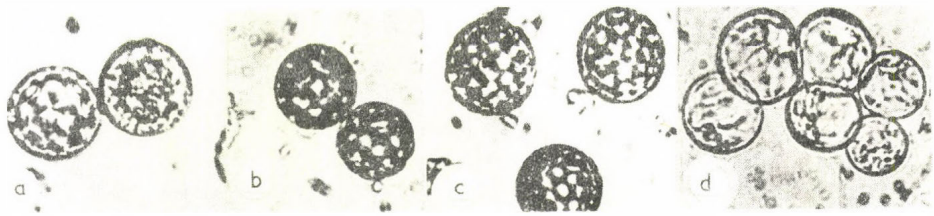

Fig.1 Isolated protoplasts

a/ from leaf mesophyll of *S. tuberosum* (2n=48)
b/ the same from a dihaploid stock (2n=24)
c/ from leaf mesophyll of *S. chacoense* (2n=24)
d/ from callus of *S. tuberosum* (2n=24-84)

parafilm-sealed Petri dishes for 18-25 days at 25°C. The cultures were illuminated daily for 16 hours (500 to 600 lux). The calluses formed (~1.0 mm^3) were cut out together with a piece of agar block and transferred to a similar medium containing no osmoticum. The calluses were cultured until they reached a size of 2 to 4 mm in diameter (10 to 14 days). Then they were transferred to an MS medium suitable for the induction of morphogenesis (macro- and micronutrients, and chelated iron as in MS, vitamin B_1 3.0 mg/l, vitamin B_6 1.0 mg/l, BAP 5.0 mg/l, NAA 1.0 mg/l, sucrose 20 g/l, agar 20 g/l at pH 5.5-5.6). A nurse-tissue technique was applied (12). The cultures were grown for an additional 12 to 14 days under identical conditions except that the light intensity was increased to 2,500 lux. Then the tissues were transferred to a low phytohormone medium (BAP 0.3 mg/l, NAA 1.0 mg/l). The shoots formed, about 3-5 mm long, were removed from the cultured tissue and rooted on a special medium developed for the rapid propagation of potato (13). The plantlets were propagated on the same medium by conventional in vitro cloning techniques and transferred to soil to study their properties. The regenerated plants were compared with typical plants of the parental species and with those of the sexual hybrid produced in 1977 (*S. tuberosum (Priekulsky ranii)* ♀ *x S. chacoense* ♂). Peroxidase isozyme and RuDP-carboxylase polypeptide patterns were investigated by polyacrylamide gel electrophoresis and isoelectric focusing, respectively. The ploidy level of the plants was studied by counting the chromosome numbers in root tips. Leaf lobe index values, the shape, dimensions and chloroplast content of guard cells were also studied and compared.

The number of fusion events in which one protoplast of both partners was involved (1+1) varied from 0.5 to 1.0 %. Multiprotoplast fusion products involving more than one protoplast of one of the parents (2+1 or 1+2 and other combinations) were not viable.

Hybrid cell lines, taking into consideration the fusion frequencies in our experiments, could be selected either by isolating the hybrid cells and culturing them in microdroplets (14) or by exposing the cultures to selective conditions permitting the development of somatic hybrids only (1, 2). To this end, the selection scheme described in Table 2 was applied. This scheme is based on: (1) the inability of cells originating from *S. chacoense* leaf mesophyll protoplasts to divide on the basal MS medium applied /these cells can, however, divide and form colonies from which plants can be regenerated on medium "A" (8)/; (2) the inability of the cells deriving from the protoplasts obtained from *S. tuberosum* callus tissue to produce shoots after division and initial organogenesis on MS medium (with this species organogenesis was found only if leaf protoplasts had been used as a starting material). The hybrid cells were expected to develop into plants on the selective medium, due to genetic complementation. Indeed, three shoots were obtained. Two of them

TABLE 2. Selection of hybrid cell lines and regeneration of plants

Solanum tuberosum L.		*Solanum chacoense* Bitt.
↓		↓
Protoplasts from callus tissue		Leaf mesophyll protoplasts
↓		↓
Murashige-Skoog medium	Fusion	Murashige-Skoog medium
↓	↓	↓
Cells	Murashige-Skoog medium	Cells
↓	↓	↓
Colonies	Hybrid cells	No sustained cell division
↓	↓	
Callus	Hybrid colonies	
↓	↓	
No morphogenesis	Somatic hybrid callus	
	↓	
	Morphogenesis	
	↓	
	Somatic hybrid plants	

were completely devoid of chlorophyll. These shoots perished upon separation from the callus tissue. The third shoot was successfully rooted and the plantlet was multiplied by vegetative cloning. The morphological characteristics (Figs. 2 and 3, Table 3), the peroxidase isozyme spectra, and the peptide analysis of the RuDP-carboxylase have shown that the plants are hybrids.

A number of hereditary traits of the plants were found to be intermediary (e.g. leaf morphology, properties of the guard cells, shape and colour of the tubers, length of the vegetative period). Some characters, like the luxurious (heterotic) plant development and the length of the stem, distinguish the somatic hybrid from both parents as well as from the sexual hybrid. The differences between the peroxidase isozyme patterns of the somatic and the sexual hybrids are worth mentioning because they might indicate the hybrid nature of the cytoplasm of the somatic hybrid.

The analysis of the polypeptide pattern of RuDP-carboxylase has shown that the polypeptides of the small sub-unit of the enzyme isolated from the somatic hybrid, correspond to the expression both *S. tuberosum* and *S. chacoense* nuclear genes. This proves that the plant is indeed a somatic hybrid of the two potato species.

The somatic hybrid has about 60 chromosomes (instead of the expected 72). We suggest that the hybrid plant is a fusion product of mesophyll protoplasts of *S. chacoense* (2n=24) and

Fig. 2 Morphology of
a) S.tuberosum var. Priekulsky rannii
b) somatic hybrid
c) S.chacoense
d) sexual hybrid (S.tuberosum ♀ x S.chacoense ♂)

callus protoplasts of *S.tuberosum*, which contain 36 chromosomes (about 15% of the callus cells were shown to contain 36 chromosomes). It is also possible that some chromosomes were lost during the culture of callus tissue obtained from the hybrid cell (1, 3).

To sum up: methods have been worked out to fuse protoplasts isolated from two potato species, select the hybrid cells, and regenerate plants from them. Some of the morphological and biochemical properties of the somatic hybrid differ from those of the corresponding sexual hybrid.

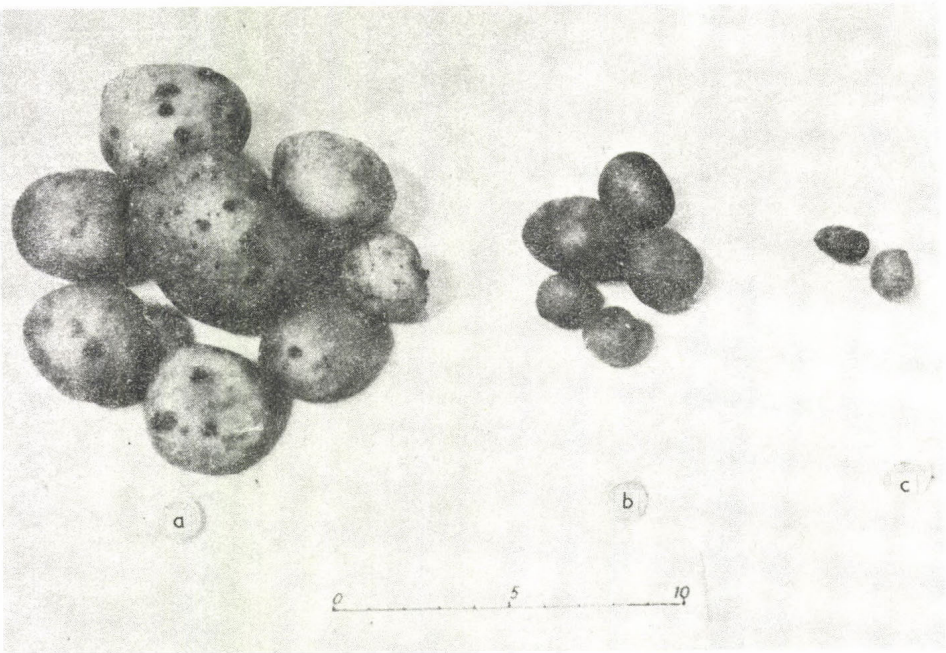

Fig. 3 Tubers:

a) S. tuberosum
b) somatic hybrid
c) S.chacoense

TABLE 3. Some morphological and biochemical properties of the parental species and the somatic hybrid

Properties	S.tuberosum	S.chacoense	Somatic hybrid
Plant	Middle sized readily lodging, multicaulis	Middle sized non-lodging, 1-2 stems	Large, non-lodging, multicaulis
Stem	Straight (up to 0.5 m high)	Straight (up to 0.6 m)	Straight, high (up to 1 m)
Leaf	Green, 3-4-paired, up to 20 cm long. Slightly dissected. Lobules are rounded. Downiness is weak, hairs are long.	Dark-green. 4-5-paired, up to 20 cm. Highly dissected, no lobules. Downiness is weak, hairs are short.	Dark-green, 4-5 paired, up to 30 cm. Highly and moderately dissected. Lobules are rounded. Downiness is weak, hairs are long.
Leaf index	1.32	2.10	1.42
Guard cell dimensions (average)	51	26	62
Tubers	White, rounded-oval, middle (up to 80 g)	White with a violet tint. Shape is diverse, very small (up to 5 g)	White with a violet tint, rounded-oval small (up to 30 g)
Starch content (%)	9.16	13.61	10.64
Sensitiveness to Pektobacterium phytophtorum	4.6	0	1.6

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INTERSPECIFIC AND INTERGENERIC SOMATIC HYBRIDISATION BETWEEN SOME SOLANACEOUS SPECIES

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ABSTRACT

Two principle methods for the selection of interspecific somatic hybrids within the genus *Datura* and of intergeneric somatic hybrids between *D.innoxia* and *Atropa belladonna* are described. First, the use of chlorophyll deficient mutants for complementation selection. The second method is based on the observation that the somatic hybrids obtained by the complementation selection show a more vigorous growth pattern in the callus stage than the non-hybrid calli. The more vigorous growth pattern of the hybrids can be used for their identification.

It is a great problem in somatic hybridisation experiments to select the hybrids after their development. Detection at an early stage of development would be advantageous.For this purpose in the last five years several selection systems have been established. Successful in early detection of somatic hybrids in addition to other methods was the combination of different biochemical mutants as auxotrophic (1) or chlorophyll deficient mutants (2) and mutants resistant to drugs (3). Also successful was the fusion of protoplasts of chlorophyll deficient mutants with wild type protoplasts which are unable to divide under the chosen culture conditions as was first demonstrated on *Petunia* (4). In this case green hybrid plants were obtained by combination of protoplasts of the green wild type of *P. parodii* which showed under the same culture condition only development into small cell clusters. The potential for regeneration of *P.hybrida* was dominant in the hybrid cells.

For applied purposes, especially in plant breeding, the use of mutants is unpractical, since normally such mutants are not easily available. Selection methods which are independent of mutants would be advantageous.It is well known that sexually produced hybrids often show a more vigorous growth pattern than the parental plants (5). We could observe on several interspecific and intergeneric somatic hybrids of the genera

Datura and *Atropa* that this heterosis effect can be expressed already in the callus stage. Perhaps this phenomenon would be a good marker for an early identification of somatic hybrids, being independent of special genetic markers. The hybrids were selected either by the combination of chlorophyll deficient mutants from which the protoplasts can be regenerated to shoots with wild type protoplasts which are unable to divide, or by the combination of such mutants with early detectable morphological markers (6, 7, 8, 9). In the following paragraphs I will describe some details of the selection of somatic hybrids by the use of chlorophyll deficient mutants and additionally I will give an outlook how presumptive somatic hybrids selected only by their vigorous growth pattern can be determined as real somatic hybrids.

Three different chlorophyll deficient mutants of *D. innoxia* from which one was tetraploid (4n=48, $7/1N_L$) and two were diploid (2n=24, A1/5a and $A7/1_S$) were used for the experiments. The protoplasts of all the mutants were enzymatically isolated from leaves of aseptic shoot cultures grown on agar medium B5 according to Gamborg et al. (10) supplemented with 1mg/l BAP. They can be regenerated in V47 medium according to Binding (11). For the isolation of protoplasts from the *Datura* species *D. stramonium* and *D. discolor* and from both the tree *Datura* species *D. sanguinea* and *D. candida* (all not crossable with *D. innoxia*) aseptic shoot cultures served as the source for protoplasts. The wild type protoplasts of these *Datura* species are unable to regenerate in the V47 medium. Aseptic shoot cultures grown on agar medium B5 of *A. belladonna* also served as the source for protoplasts. But in this case only a low % of their protoplasts is able to start divisions and form calli. The fusion of the protoplasts was undertaken as described earlier (6, 7, 8, 12).

Hybrid calli were selected after transfer of developing cell clusters to agar medium B5 supplemented with 1mg/l BAP. Calli with a size of 1-2mm originating from wild type protoplasts of *D. innoxia* became green after 30 days of culture on this medium (12). Calli originating from fusions between a chlorophyll deficient mutant of *D.innoxia* and of the wild type protoplasts of one of the mentioned other *Datura* species behaved similarly. Since the green wild type protoplasts of the used species cannot regenerate, consequently green calli could be picked out as presumptive hybrids. They were transferred to fresh agar medium B5 for further cultivation. Since a low yield of the wild type protoplasts of *A. belladonna* are able to regenerate in the V47 medium another selection method must be used which is based not only on the green colour of the somatic hybrids. Simultaneously with the appearance of the green colour of the wild type protoplasts of *D. innoxia* a production of hairs can be observed, whereas *A. belladonna* shows no hairs at any developmental stage. The selection of somatic hybrid calli was possible by their green colour coded by the genome of *A. belladonna* and by the production of hairs coded by the genome of *D. innoxia (8).*

In all cases where protoplasts of a chlorophyll deficient mutant of *D. innoxia* were fused with the wild type protoplasts of one of the above mentioned *Datura* species, several green calli could be selected. Also, somatic hybrid calli showing a green colour and additionally the production of hairs were found after fusion and regeneration of protoplasts of chlorophyll deficient mutants of *D. innoxia* and of the wild type of *A. belladonna*. The chromosome numbers ranged from tetraploid to octoploid, but several aneuploid somatic hybrid lines could also be detected. A very interesting observation was made during selection of the somatic hybrids. Most of the hybrid calli especially the euploid hybrids showed a more vigorous growth pattern than the non-hybrid calli which had developed synchronously (Fig. 1). This cannot just be the result of the higher ploidy level, since this phenomenon was not observed amongst intraspecific somatic hybrid calli developed from fused protoplasts of two different chlorophyll deficient mutants of *D.innoxia*.

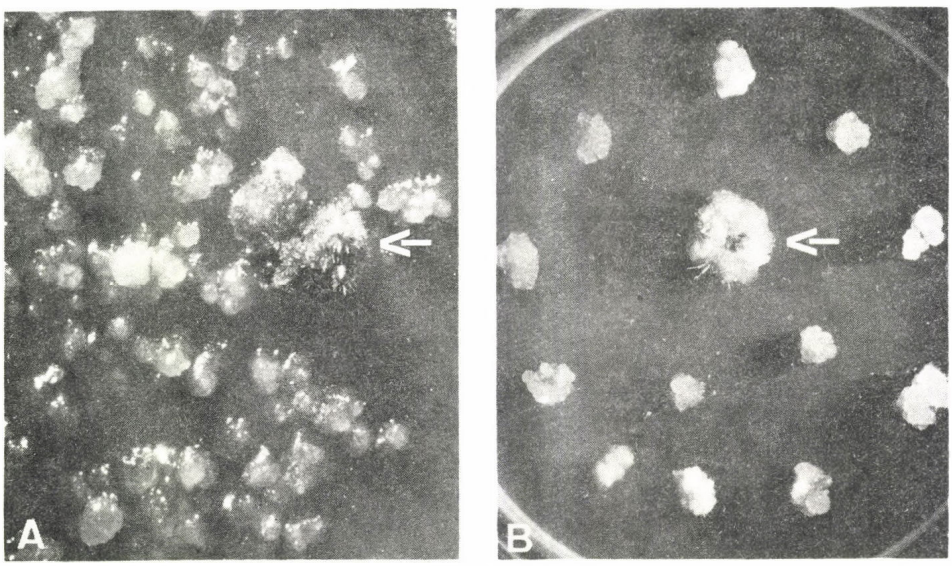

Fig. 1. Somatic hybrid calli of *D.innoxia* + *D.sanguinea* (A) and *D. candida* (B), respectively, surrounded by smaller calli of *D. innoxia*

Plants which could be regenerated from tetraploid somatic hybrid calli of *D.innoxia* + *D. stramonium* and *D. discolor*, respectively, also show a more vigorous growth pattern. They are fertile and produce fruits and viable seeds. Out of the seeds plants could be obtained which show in most cases the phenotype of the original produced somatic hybrids. The amphidiploid somatic hybrids of *D. innoxia* + *D. sanguinea*

are unable to produce roots. Moreover, shoots grafted in the greenhouse show often tumorous tissue producing teratomatas. Calli of these somatic hybrids are auxin autotrophs similar to those described for some sexual hybrids of the genus *Nicotiana* (13). The somatic hybrids *D. innoxia* + *D. candida* are able to produce roots, but they are not yet flowering. Further investigations concerning fertility and viability of the seeds must be undertaken. During culture of more than ten months the somatic hybrids of *D. innoxia* + *A. belladonna* were not able to produce well developed shoots. Only fleshy leaves could be regenerated. But after that time some of the somatic hybrid lines started to form roots and embryoids. These facts stimulate optimism to get plants in the future.

In the beginning I mentioned that the observed vigorous growth pattern of the interspecific somatic hybrid calli could perhaps be used as a marker for the selection of the hybrids. The selection of the calli growing best should deliver predominantly somatic hybrids. Of course, heterosis cannot be expected in all combinations, but in such combinations where it is the case, one is independent of other markers e.g. chlorophyll deficiency. A selection system which is based on the vigorous growth pattern of the somatic hybrids facilitates the possibility to combine somatic hybridisation with classical breeding methods as proposed by Wenzel et al. (14) for the potato. But the selection of calli which show a more vigorous growth pattern can only be the first step in finding the hybrids. One should expect that together with hybrid calli, non-hybrid calli will also be selected. To avoid the regeneration of all the selected calli to complete plants, additional screening methods are necessary. In cases where protoplasts of species which possess a different size of their chromosomes are fused it is relatively easy, by investigations of mitosis, to clarify whether the selected calli are hybrids or not. This is, for example, the case in somatic hybrids of *D. innoxia* + + *A. belladonna*. But in most cases a different size of the chromosomes of the parental plant species used is not present. Other methods for the final identification of the hybrids must be employed.

Power et al. (3) introduced isoenzyme investigations for distinguishing somatic hybrids. Such investigations seem to be an excellent method especially in cases where the somatic hybrids are selected only by their vigorous growth pattern. For the genus *Datura* we have found that the amylases seem to be the best markers for the identification of somatic hybrids (15). Aseptic shoot cultures of the *Datura* species and the somatic hybrids mentioned above were taken for the preparation of enzyme extracts. Separation of the enzymes was made with acrylamide gels in the presence of starch. After electrophoresis the gels were kept for 1h in a moist chamber to allow the amylases to digest the starch. After that time the gels were transferred to I_2 solution. Transparent bands indicated amylase activity. The different *Datura* species possess, in most cases, only one transparent band representing amylase

activity, except *D. sanguinea* and *D. metel* which possess a second but weak band. In most cases the electrophoretic pattern of the amylases of the different species is not identical. In the four somatic hybrids, two transparent bands representing amylase activity were always observed. In each somatic hybrid line investigated one of these two bands was identical with that of the corresponding parental plant species (Fig. 2) used for the hybridisation experiments.

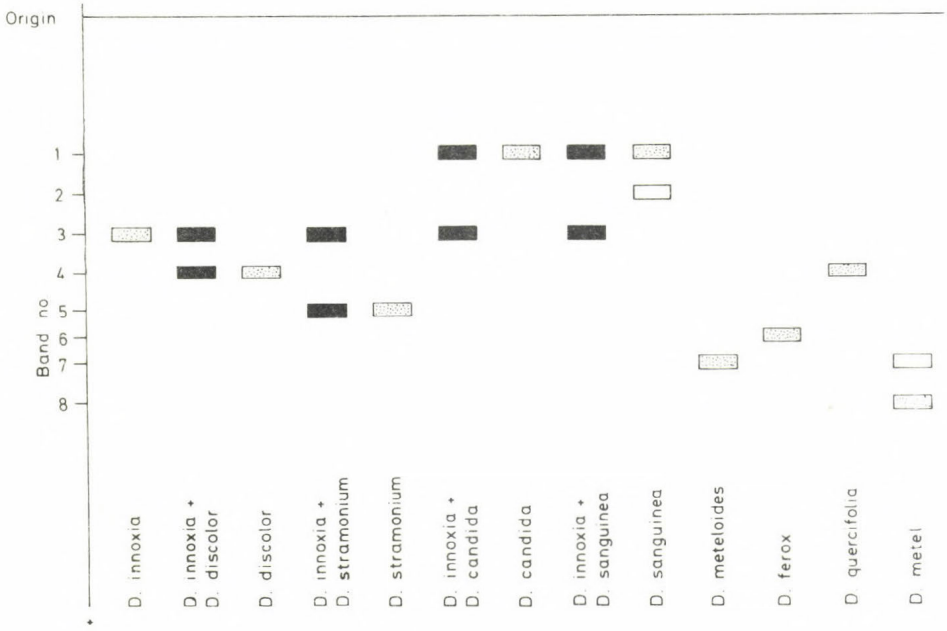

Fig. 2. Acrlyamide gel pherogram of the amylases from several *Datura* species (punctated graphs represent fully expressed bands and white graphs weak bands) and from four somatic hybrids (black bands).

We have already started experiments to select somatic hybrids of the genus *Datura* using the vigorous growth pattern as a marker. Investigations of the amylase isoenzymes of the selected calli as the second step of selection shall serve for the final identification of the somatic hybrids.

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INTERGENERIC TRANSFER OF NUCLEAR MARKERS THROUGH FUSION BETWEEN DIVIDING AND MITOTICALLY INACTIVE PLANT PROTOPLASTS

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ABSTRACT

Green plants have been redifferentiated after fusion of albino carrot (*Daucus carota*) protoplasts with inactive leaf protoplasts of *Aegopodium podagraria* and *Petroselinum hortense*. Characterization of plants having carrot karyotype (2n=18) has revealed the transfer of nuclear markers from the inactive partners into the albino carrot. Gene transfer via fusion of dividing and mitotically inactive protoplasts will be discussed as a method for genetic manipulation of higher plants.

INTRODUCTION

In recent years significant progress has been made in somatic hybridization of higher plants via protoplast fusion. Parasexual integration of complete parental genomes has been successfully achieved in different interspecific (1-7) and intergeneric (8-9) fusion combinations. Several attempts at cell fusion, however, have failed to result in hybrid plants (10--13). These observations, as well as the results of cytological studies on hybrid cell lines (14) indicate an expression of somatic incompatible responses at various stages of somatic hybridization. Considering a possible effect of incompatible reactions, reduction in the size of the introduced genome can be proposed as an approach to bypassing genetic barriers acting in fused somatic cells. The techniques have not yet been developed for genetic manipulation with isolated nuclei (15), plant chromosomes or genes. The method of protoplast fusion, however, offers various alternatives for introducing only part of the plant genome. In this paper we suggest the fusion between dividing and mitotically inactive protoplasts to transfer nuclear markers.

METHODS

The plant material and all details of the techniques of protoplast isolation, fusion and culture have been described previously (7, 16, 17).

RESULTS

Characterization of the albino *Daucus carota* mutant used as active fusion partner

In the present somatic hybridization experiments, the selection system has been based on genetic complementation of an albino carrot mutant found in an M_2 plant population after treatment of seeds of "Nantaise Slendero" carrot variety with ethylmethansulphonate (EMS). The suspension cultures established from the white seedling were highly embryogenic. The albino phenotype was maintaned in redifferentiated plants cultured on complete medium (7). In the leaves of these albino plants, the plastids were undifferentiated and failed to develop a lamellar system. The mutant did not synthesize chlorophyll and the lack of protochlorophyllide accumulation in dark grown leaves indicated a block in chlorophyll biosynthesis. An increased formation of carotenoids with α-ionon rings was detected in albino leaves (16).

The genetic characterization of this mutant has been performed by segregation analysis of sexual progenies of somatic hybrids between this albino *D.carota* (2n=18) and *D.capillifolius* (2n=18) (7). The observed segregation (34 green : 1 albino) indicated the nuclear determination of mutation. Extensive studies have been carried out to test the stability of this albino mutant. Summarising recent results we have found no green revertants from $2x10^8$ albino protoplasts. Albino cell cultures were also retreated with 0.2 % EMS solution for 24 hours. After 2 weeks expression period, protoplasts ($7.9x10^6$) were isolated and cultured. The EMS treatments failed to induce back mutation in regenerated embryos. The lack of spontaneous and induced reversion and simultaneous alteration of various biosynthetic pathways indicates that this mutation might be a deletion.

Fusion with non-dividing *Ae.podagraria* leaf protoplasts

Plants have been regenerated from green calli developed after intergeneric protoplast fusion of albino *D.carota* and normal green *Ae.podagraria* (16). Despite the fact that the selected plants (designated CAPO plants) possessed *D.carota* chromosomes (2n=18) certain *Ae.podagraria* markers were retained in these plants. On the basis of control experiments indicating the lack of back mutation in albino protoplasts, the green phenotype in selected plants was supposed to be the result of genetic correction by the *Ae.podagraria* parent. Since, the albino phenotype is the result of nuclear mutation and in the selected plants no segregation or variegation was observed it was suggested that nuclear complementation was responsible for restoration of greening. However, cybrid formation via maintenance of introduced *Ae.podagraria* chloroplasts could not be completely excluded. Therefore, the chloroplast DNAs have been characterized by their EcoRI restriction pattern (17). The pattern of EcoRI fragments of chl-DNAs from CAPO plants and

from *D.carota* were found to be identical. The detection of ultrastructurally normal carrot chloroplasts in CAPO plants provided an additional indication for nuclear complementation.

Further characterization of regenerated green plants revealed the appearance of *Ae.podagraria* specific markers in root development and in root carotenoids. Deviation from the typical carrot root development has been observed on young CAPO embryos grown in hormone free medium (Fig. 1). At a later stage, shoots

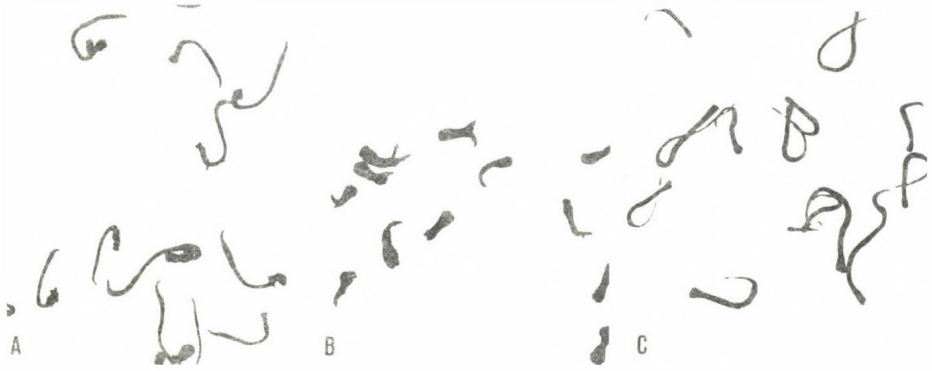

Fig. 1

Root development of young embryos

A./ Albino mutant B./ CAPO C./ Normal carrot

and plantlets have frequently been differentiated from the tap roots of CAPO plants on hormone free medium. Plant regeneration from roots was not observed on carrot plants cultured under the same conditions. Like the *Ae.podagraria* plants, the CAPO plants formed white roots in which neurosporene was accumulated in considerable quantities (16). Similar neurosporene accumulation has not been detected in white roots of somatic hybrids between the same albino mutant of carrot and *Daucus capillifolius*. This indicates that the enhanced neurosporene accumulation in CAPO plants is determined by the *Ae. podagraria* partner.

Because of the abnormalities in plant development and in flower-formation, the CAPO plants were not able to produce sexual progeny. In order to reduce the observed defects somatic back hybridization is being carried out by fusion between non-dividing CAPO leaf protoplasts and cell culture protoplasts of albino mutants. The first redifferentiated green plants were found to be more viable (Fig. 2). These plants could be transplanted into the soil. It is hoped that after a sexual cycle, the genetic analysis of CAPO plants can be completed.

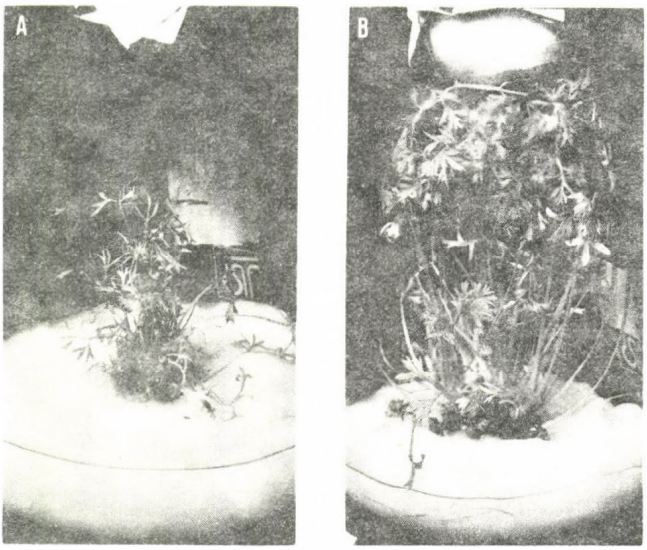

Fig. 2

Phenotype of a flowering CAPO plant (A) and of a plant regenerated after back hybridization with the albino mutant (B)

X-ray irradiated leaf protoplasts of *Petroselinum hortense* as nuclear donors

In further studies on inactive nuclei-mediated complementation the protoplasts of the albino mutant ($4x10^5$) were fused with leaf protoplasts of *Petroselinum hortense* ($2x10^5$). To induce the elimination of the inactive genome, the leaf protoplasts were exposed to X-ray irradiation (9Kr) prior to fusion. The two green calli isolated after the fusion had the same chromosome number as carrot (2n=18). After a prolonged culture period on inductive - hormone free - medium, plantlets with small leaves have been differentiated from calli of one of the selected lines (Fig. 3).

These plantlets were significantly different from the previously described CAPO plants. In primary cell cultures initiated from the leaves of the regenerated plants embryogenesis was very poor. The pattern of chloroplast DNA fragments after EcoRI digestion showed the presence of carrot chloroplasts in selected green calli (17). This finding suggests nuclear complementation instead of restoration of photosynthetic apparatus through maintenance of *Petroselinum hortense* chloroplasts.

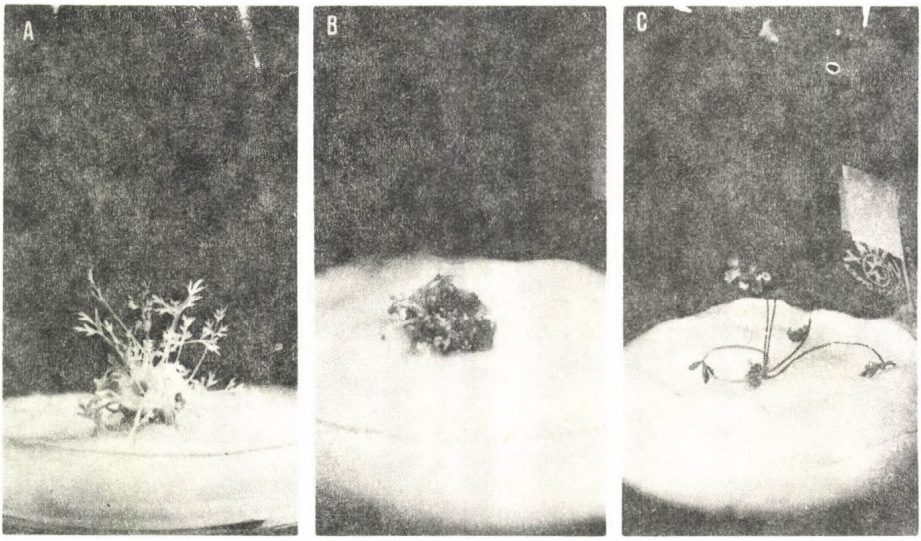

Fig. 3

Morphology of a green plantlet (B) redifferentiated after fusion of protoplasts of albino *Daucus carota* (A) and *Petroselinum hortense* (C)

The further detailed characterization of the redifferentiated plantlets has been hindered by the high lethality of the differentiating tissues.

DISCUSSION

The presented data show two examples of restoration of the green phenotype in a stable albino carrot mutant by transferring nuclear markers through fusion of mitotically inactive protoplasts. In both intergeneric fusion combinations, the regenerated green plants possessed carrot chromosomes (2n=18), while the phenotypic effects of certain genes from the inactive partners could be detected. In contrast to the somatic hybrids produced previously, the data described here indicated the elimination of one parental genome - the inactive one - although characters have been introduced into the albino carrot mutant. In the present experiments with leaf protoplasts the elimination process has been accelerated by fusion of inactive or X-ray irradiated protoplasts. Irradiation of one fusion partner has been successfully applied for induction of directional chromosome elimination in fused mammalian cells (18) and in transferring cytoplasmic male sterility by protoplast fusion (19).

The phenomenon of cell fusion mediated gene transfer is known

from the results of fusion experiments with mammalian cells. Studies on the fate of inactive nuclei of chick erythrocytes in fused cells revealed that if the chick chromosomes underwent premature chromosome condensation (PCC) they were readily lost at mitosis, although chick specific gene products were verified in these hybrids that contained no detectable chick chromosomes (20-23). Experiments on transferring genes by means of isolated metaphase chromosomes frequently resulted in cell lines expressing new markers in the absence of additional chromosomes (24-28).

We suppose that in the present study interphase nuclei of inactive leaf protoplasts served as genetic donors after structural changes caused by interaction between nuclei in heterokaryons. Cytological analysis of fused plant cells showed mitotic activation of non dividing nuclei by the active partners (29, 30). Occurence of PCC has been recently demonstrated after fusion of mitotic and interphase plant protoplasts (31). Pulverization of interphase rice or wheat nuclei was observed three hours after fusion with mitotic wheat protoplasts. If condensation of chromatin is preferentially induced by mitotic partners the frequency of hybrids with genetic constitution described in the studied fusion combinations depends on the number of mitotic protoplasts taking part in fusion events. The recently applied methods of protoplast isolation do not support the release of protoplasts in mitosis. Further development is required in methods for isolation and fusion of synchronized plant protoplasts to determine the conditions under which protoplast fusion mediated gene transfer can be realized with an increased efficiency.

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TRANSFER OF GENETIC INFORMATION IN HIGHER PLANTS VIA PROTOPLAST FUSION

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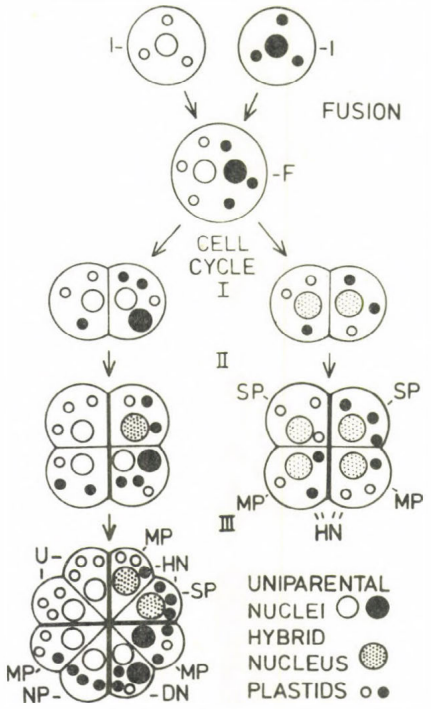
ABSTRACT

Processes which occur in proliferating fusion products of plant protoplasts are discussed. Segregation, elimination and fusion of cell organelles and genophors, and recombination of genophors, are considered. Some details are dealt with on the loss of chromosomes and the stabilization of karyotypes in fusion products of Vicia faba + Petunia hybrida.

Reports on asexual transfer of genetic information in pro- and eukaryotic systems have been increasing rapidly within the last few years. Besides sexual reproduction, protoplast fusion is still the only efficient way of combining genetic informations of higher plants. Especially interesting in genetics and plant breeding are those recombinant clones which contain predominantly monotypic genomes with a limited number of foreign genes. The genesis of defined heterozygotic fusion products is most easily achieved by the fusion of protoplasts which carry largely identical genomes. This is the case in intra- and some interspecific hybrids.

The combination of genetic material of less closely related plants is an especially fascinating aspect of experiments on asexual recombination. At first sight, protoplast fusion seems not to be an appropriate tool for producing vital clones because the fusion body is extremely heterogeneic. The solution to this problem has been attempted by the fusion of subprotoplasts (1), by inactivating the nuclei of one protoplast type before fusion (2), and by enucleation of protoplasts by the action of cytochalasin and fusion of the miniprotoplasts to complete ones (3). The intention of these experiments was to eliminate special organelle types before fusion. Organelles are also eliminated in proliferating cells, as has been extensively discussed earlier (4). Imaginable events in the fusion products are illustrated in a rather simplified way in Figure 1.

Beginning segregation and elimination of plastids (1,5,6) and nuclei (6) has been found in young fusion products. The



Assumptions: on the left, the first mitosis of the dark nucleus delayed; on the right, hybrid nuclei formed during the first synchronized mitosis.

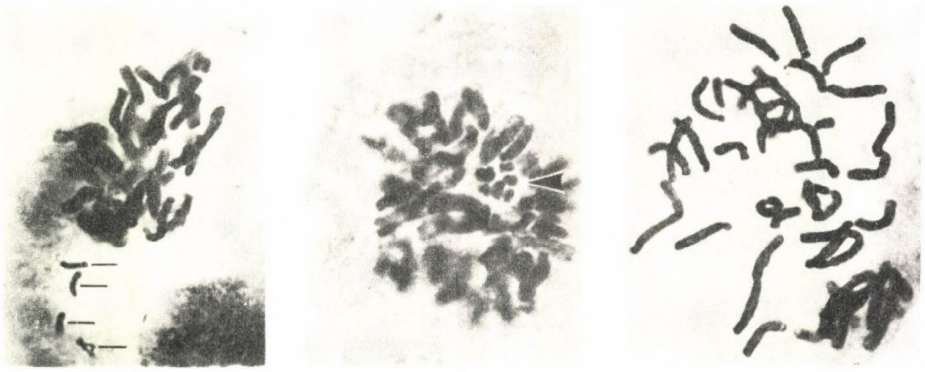
The plastids are assumed to propagate at equal rates.

I - isolated protoplast
F - fusion body
U - uniparental constitution
DN - heterokaryon
HN - hybrid
NP - nucleus-plastid recombinant
MP - mixed plastid population
SP - plastid segregant

Figure 1. Imaginable fates of nuclei and plastids in fusion products.

probability of the regeneration of chimeric plants is reduced even in intrageneric hybrids (7) by segregation of the various resulting clones, but also by their different growth characteristics. New combinations of nuclei and mitochondria have also been obtained in regenerated plants (2,8). Hitherto, the transmission of complete cell organelles to the cell progeny of fusion bodies has been briefly discussed. Most efficient reduction of the genetic complexity is achieved if recombination of different types of cell organelles by segregation and elimination is associated with recombination processes between foreign organelles of one type. Recombination of mitochondrial genes has been taken into consideration to explain different degrees of cytoplasmic male sterility and malformations of flowers (9). No indication has been obtained for genetic interchanges between the plastids of higher plants. Especially interesting alterations with respect to the reduction of the complexity of heterogeneic fusion products are loss of chromosomes and translocation of chromosomal segments. These events may lead to cells in which just one segment of a foreign chromosome is left together with complete genomes of one partner. Preferential elimination of chromosomes of one parent has been found in somatic hybrids of Glycine max + Nicotiana glauca (10). Two hybrid clones of Vicia faba + Petunia hybrida have been discovered in cytological investigations of contaminated populations of 50-day old calluses, which contained nuclei of Petunia type and,additionally, only

one or two Vicia chromosomes mostly located in the cytoplasm (6). The third hybrid cell line of Vicia + Petunia (P77-11-7) has now reached an age of more than two years. Chromosome numbers were recently determined in 30 subcultures. The values point to a stabilization of the numbers of Vicia chromosomes at 17-18 and multiples of these numbers (Figure 2a, b). 0-4 Petunia chromosomes were present in cells of the low ploidy level. The same karyotypes (besides others) had been found already in two--month old cultures. The highest number of Petunia chromosomes, 12, was visible in a mitotic cell which contained about 72 Vicia chromosomes. The Petunia chromosomes frequently appeared to be concentrated in a certain area of the metaphase plate. Fewer chromosomal aberrations have been found in recent examinations than about one year ago. Some mitotic figures, however, exhibited chromosome fragments, anaphase bridges and one dicentric chromosome. It seems likely that the number of Vicia chromosomes of 17-18 represents a true triploid level. This suspicion is supported by the presence of three satellite chromosomes. The other chromosomes are not differentiated in preparations stained with acetocarmine. Analyses by the Giemsa C-banding technique (11) have sofar revealed no satisfactory results.



a b c

Fig. 2 a and b: Mitotic figures of the Vicia faba + Petunia hybrida hybrid clone P77-11-7 with 34-36 chromosomes of Vicia and 4 (a) and at least 7 (b) chromosomes of Petunia.
c; A mitotic figure of a callus of Vicia faba regenerated from the experiment P77-11, containing 36 chromosomes.

The question arises whether certain features found in fusion products are peculiar for this system or whether adequate events are found in uniparental clones under equal conditions. This question has been tackled with respect to the Vicia karyotype in the clone P77-11-7. As a reference, the karyotypes of protoplast derived clones of Vicia faba have been determined (Table 1). The high proportion of calluses of which the chromosome numbers can be reduced approximately to

TABLE 1 Chromosome numbers in callus clones of Vicia faba

Clone	Chromosome Numbers									
1	12!		24!							
2	12!		24!							
3	12!		24?							
4	12		24!							
5			24!							
6			24!							
7			24!							
8			24?							
9			24!		48					
10			24!		48?					
11					48?					
12					48?				96	
13									96!	
14									96?	
15	12	16,17!		32?						
16		16								
17		18!		36!						
18		17!		34?						
19		20!		40?						
20				36!						
21				36!			72!			
22								140-150!		
23			29!			56!	85-90			
24		24!			48!					
25		24!			48!					
26		24!			48?					
27		24?			48?					
28		24!		36!						
29				34!						

Numbers of calluses with chromosome numbers which fit to ploidy levels of

2n	3n±2	4n	5n-1	6n±4	8n	10n-4	12n	15n	16n	24n
5	5	15	1	8	8	1	1	1	3	1

Table 1. The clones No. 1-23 were 24 months old, the others 20 months. - (?) numbers are based on approximative values; bare numbers refer to not more than four calculations at least one being a precise value; (!) numbers have exactly been determined in 1-6 cells and were supported by a total of 5-30 figures.

a triploid level, as well as the occurrence of triploid callus grown from an explant of a diploid Vicia faba embryo (12) strongly suggest that the triploidy in clone P77-11-7 reflect-

ed a common tendency of Vicia faba callus (Figure 2c).

It is obvious from the presented data and considerations that such complex marker system as chromosome morphologies, and also patterns of proteins (cf. 13) or of DNA cleavage products (9) are needed for analyses of largely heterogeneic fusion products. It is also evident that desired recombinants will be achieved only if their growth and selection is favoured.

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FUSION OF CITRUS AND TOBACCO PROTOPLASTS - A NEW SYSTEM FOR SOMATIC HYBRIDISATION STUDIES WITH REMOTE SPECIES

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ABSTRACT

A two-step procedure for somatic hybrid selection based on differential phytohormone requirements of the parental species, *Citrus* and tobacco, is described. The presence of well-defined markers, enabling selection, identification, and characterisation of somatic hybrids, suggests the use of these species for intergeneric somatic hybridisation experiments.

INTRODUCTION

The use of polyethylene glycol as a potent inducer of protoplast fusion (1,2) has laid the basis for the rapid expansion of the field of somatic hybridisation. Hybrid cell lines have been recovered from various intergeneric combinations (see ref. 3 for list). Morphogenesis has led to intergeneric hybrid plants with "normal" morphology in the case of *Solanum tuberosum* * *Lycopersicon esculentum* (4). Somatic hybridisation of more distantly related species may lead to the formation of monsters with distorted morphologies and various degrees of structural and functional abnormalities, as was exemplified with *Arabidopsis-Brassica* somatic hybrids (5).

While some of the intra- and interspecies somatic hybrid plants allow scope for agricultural (potato), horticultural *(Petunia)*, or pharmaceutical *(Datura)* applications, intergeneric somatic hybrid cell lines hold potential for somatic-cell-genetic studies in higher plants. These would deal with nuclear and chromosomal behaviour, recombination and segregation, chromosome mapping, differential gene expression and various aspects of metabolic regulation, differentiation, and morphogenesis - to mention only a few. Work towards these ends is facilitated by the use of appropriate plant material, expressing various markers at various levels, thus enabling the identification of somatic hybrid cell lines and the study of the differential expression of these characters.

In this communication, we describe a system of phylogenetically remote species, *Citrus* (Rutaceae) and *Nicotiana* (Solanaceae), for somatic hybridisation experiments.

MATERIALS AND METHODS

Plant material. The plant material used in our studies was chosen because of availability, culturability of their protoplasts, and the presence of well-defined markers allowing both selection and identification of somatic hybrids. For the one parental species we used *Nicotiana tabacum* SR1, a non-mendelian streptomycin resistant mutant obtained by Maliga et al. (6). Morphogenetic mutant calluses and plants are resistant to 250 mg/l streptomycin. Mesophyll protoplasts, both mutant and wild types, were able to grow on 20 mg/l streptomycin but were both killed at higher levels of the drug (Harms, unpubl.). The expression of resistance is therefore tested favourably on morphogenetic cultures. Using NT medium (7) containing 4 mg/l CPA and 1 mg/l kinetin, SR1 mesophyll protoplasts were cultured routinely with high efficiency (8). No growth on medium containing 1-5 % galactose or lactose was observed either with SR1 protoplasts or SR1 calluses.

For the other parental species we used *Citrus sinensis* var. Shamouti. Embryogenic ovular callus lines from this species have been established in 1972 by Kochba et al. (9). These cultures are white, globular, and higly friable. It is also a characteristic feature of this material that it grows in the absence of exogenous hormones and also on galactose and lactose media. Somatic embryos can be induced by various treatments (10, 11). Protoplasts isolated from callus (12), submersed callus and suspension cultures (13) of both embryogenic (L5) and non-embryogenic (L10) lines grow in the absence of hormones - the only reported example of protoplasts which can do so.

Fusion. *Citrus* cells and protoplasts often contained prominent starch grains. These and the dense cytoplasm of the *Citrus* protoplasts together with the functional chloroplasts of the SR1 mesophyll protoplasts provided markers for the visual distinction between heterokaryocytes and parental protoplasts after fusion and during the initial stages of development. The protoplasts were fused using polyethylene glycol and, after diluting and washing, plated in liquid NT medium (4 mg/l CPA, 1 mg/l kinetin). For controls, both parental protoplast types were cultured separately, untreated as well as PEG-treated, and also co-cultured (unfused mixtures of tobacco and *Citrus* protoplasts) in the same medium.

Selection. The selection procedure devised for the recovery of somatic hybrids from fused mixtures of *Citrus* and tobacco protoplasts was based on their different requirements for exogenous phytohormone supply. Control experiments performed beforehand showed an absolute requirement for auxin and cytokinin of the SR1 protoplasts. In contrast, *Citrus* protoplasts not only grew autotrophically in the absence of exogenous hormones, but their growth was also remarkably inhibited in the presence of hormones, as was shown previously (14) and confirmed in our experiments (13).

A two-step selection procedure was therefore applied (Fig. 1): tobacco-*Citrus* fusion mixtures were first plated on hormone$^+$ medium known to inhibit growth of parental *Citrus* protoplasts, and, hopefully, also of the somatic hybrids. Emerging colonies were then transferred to hormone-free medium to eliminate parental tobacco colonies and to select for somatic hybrids able to grow in the absence of hormones. Colonies passing the selection were cloned for further analysis.

	tobacco	somatic hybrid	*Citrus*
1.step hormone$^+$	growth	growth	no growth
2.step hormone$^-$	no growth	growth	-
3.step:	-	clone analysis	-

Fig. 1 Selection scheme for the recovery of somatic hybrid colonies after fusion of *Citrus* and tobacco protoplasts.

RESULTS AND DISCUSSION

Heterokaryocytes, as recognised microscopically through the presence of parental visual markers (Fig. 2a) and differential nuclear staining (Fig. 2b), were readily formed upon fusion and most of them remained viable at least for several days, as was obvious from the development of cytoplasmic strands and the progressing mixing of the parental cytoplasms. Cell wall formation and first divisions (Fig. 2c) leading to small cell clusters were observed in some instances. The presence, in the cells of such clusters, of the visual markers of both parental species suggests their hybrid nature. It also indicates that there seem to be no stringent cellular incompatibility mechanisms acting against the formation of *Citrus*-tobacco hybrid cells at this stage, although it is not known whether or not these cells were truly hybrid with respect to their nuclear constitution.

Upon transfer to hormone-free medium, colonies derived from parental tobacco protoplasts turned brown and stopped growing, as did the colonies from tobacco control cultures. In a first series of fusion experiments, 196 clones were selected, 126 of which grew moderately on hormone-free medium during the first two passages (8 weeks) but ceased growing thereafter, while 70 clones grew well on selective medium and only moderately on medium containing hormones (Fig. 2d). These were used for further analysis. A considerable number of pure *Citrus*-type colonies were also recovered on hormone-free medium, a result not

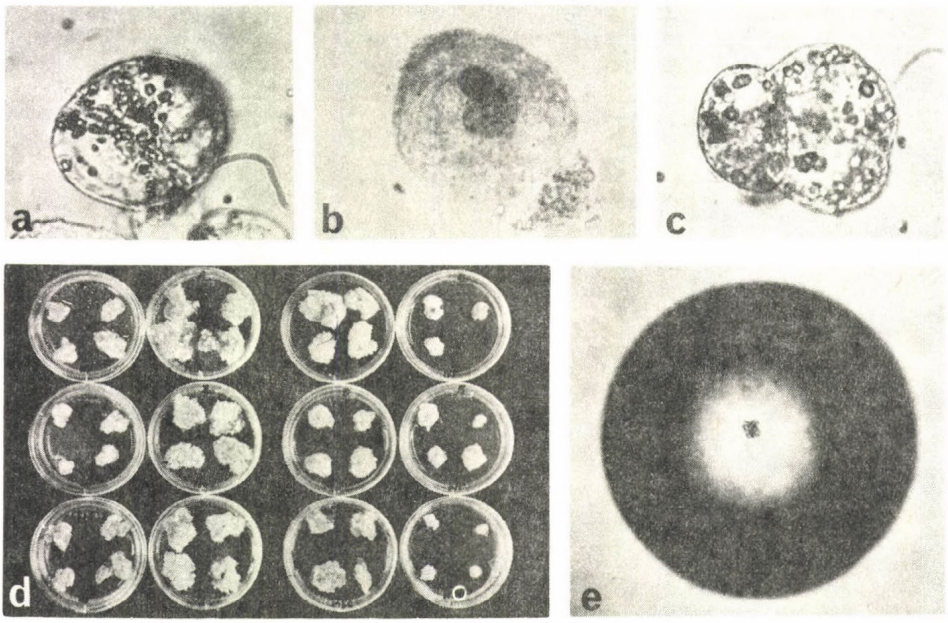

Fig. 2. Culture of *Citrus-Nicotiana* fusion products
a - heterokaryocyte after 3 days of culture
b - differential nuclear staining (propionic orcein)
c - division of *Citrus-Nicotiana* fusion product
d - growth performance of some of the selected presumed hybrid clones on hormone$^+$ (outer rows) and hormone$^-$ medium (inner rows)
e - hand-picked colony growing in a 5 µl hanging droplet

expected according to the data from the previous control experiments. The same response was also observed in control cultures from unfused mixtures of *Citrus* and tobacco protoplasts, indicating a cross-feeding-like phenomenon. These findings prompted us to investigate the effects of co-culturing in more detail. These studies and their significance for somatic hybrid selection are reported elsewhere (13).

Chromosomal analysis of some of the selected clones was performed but did not provide doubtless evidence for their somatic hybrid nature. As a matter of fact, metaphases were hard to find in the callus material. The chromosome number of tobacco-*Citrus* hybrids was expected to be more than 102 rather than 66 (*N. tabacum*, 2n=48 + *C.sinensis*, 2n=18) since chromosome counts of the parental *Citrus* cultures showed they were mixoploid, containing 54-72 chromosomes, but they had retained their embryogenic capacities.

Growth analysis was performed with subclones of the original 70 selected clones on medium containing 2 % galactose or lactose and also on selective medium containing 250 mg/l streptomycin. None of the subclones grew on galactose or lactose, but all of them were streptomycin resistant.

So far, 24 of the 70 selected clones were also analysed for peroxidase, acid phosphatase, and esterase isozymes using isoelectric focusing (IEF) on polyacrylamide (PAG) slab gels (pH 3.5-9.5). With all isozymes studied, the IEF banding patterns of the clones were consistent with SR1 tobacco controls and showed no additional bands that could be attributed to the *Citrus* parent.

The experimental analysis of some of the selected clones from the first series of fusion experiments has provided no conclusive evidence for their presumed somatic hybrid nature. While the data support the view that the selected material is of parental tobacco nature, the growth of these clones on hormone--free medium was astonishing. Adaptation to hormone$^-$ condition in a way similar to habituation would provide a possible explanation. It is interesting to note, however, that such colonies did not arise from the tobacco control cultures.

Since however, small cell clusters were observed which carried mixed parental characters, it seems reasonable to assume that they ceased growing at a later stage and therefore could not be recovered during the selection. From this it seems likely that *Citrus*-tobacco hybrid cells have the hormone requirements similar to those of tobacco rather than exhibiting the hormone autotrophy of *Citrus*. While it had been hoped that somatic hybrids would express both characters under respective selective pressure (Fig. 1), such behaviour could not be predicted a priori. The data presented rather speak in favour of non-expression of hormone-independent growth and dominance of hormone auxotrophy, possibly as a result of the first selection step on hormone$^+$ medium.

From a second series of fusion experiments we have also recovered a number of colonies which passed the described selection procedure (4 subcultures on hormone$^-$ medium). The morphological appearance of some of these clones is very much different from the ones previously selected. Analysis of this material, now being in progress, may reveal supportive or disproving evidence for their presumed hybrid nature, and may finally demonstrate that *Citrus*-tobacco somatic hybrids can be obtained using the selection system outlined in this communication. The culture of isolated hybrid colonies has been suggested and used to recover somatic hybrids of soybean-*Nicotiana glauca* (15) and of *Arabidopsis-Brassica* (16). As an alternative approach to selection systems based on physiological complementation, we also started work on the hand-picking and subsequent culture of individual fusion products (Fig. 2e) following their visual identification through the presence of cellular markers from both parental species. Using this approach, we hope to learn more about the behaviour and growth requirements of hybrid cells between phylogenetically remote species.

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PROTOPLAST TECHNIQUES INCORPORATED INTO APPLIED BREEDING PROGRAMS

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ABSTRACT

The presented model describes how microspore and protoplast techniques can be combined with classical breeding approaches in applied potato breeding programs. Starting with autotetraploid clones, the dihaploid level is achieved parthenogenetically. Subsequently the dihaploids are subjected to microspore culture and in vitro chromosome doubling to obtain the homozygous-condition. Such dihaploids are crossed to yield well defined F_1 hybrids, now possessing characters from different starting clones. In a final step, their genomes and plastomes are combined by somatic hybridization to a completely heterozygous tetraploid plant. As potatoes are propagated vegetatively, the fusion product can become a stable variety immediately. For selection of the fusion products colour markers and hybrid vigour combined with isoenzyme investigations are used. Besides this breeding scheme further approaches in applying protoplast techniques in plant breeding are summarized.

INTRODUCTION

For nearly ten years excellent reviews have outlined the marvellous potential of tissue culture techniques and the use of protoplasts in particular for solving the world's food problems (1-4). As a result, funds could be activated for this type of research and as a second consequence institutions which have sponsored something fantastic are today expecting overwhelming results. However, higher plants are not as swift growing as microbes and plant breeding, even at the single cell level, is a time-consuming task. So we still have an excuse for the lack of new and better test tube varieties. But the future does not look too promising either, for instance if one focuses on cereals, where even first steps in an applied direction failed. Therefore, one has to choose a more pragmatic approach. A few crops exist where protoplast regeneration became possible, and where in vitro derived lines are already grown in the field in concurrence with plants grown from seeds (Table 1, Fig. 1). The number of such examples increases when important drug plants are included, and the picture becomes more colourful when horticultural plants

are taken into consideration as well. I will restrict myself predominantly to *Solanum tuberosum* the major food crop where some in vitro culture prerequisites could be achieved recently (13-16). The potato is a pragmatic choice as its acerage is going down and also our program is far away from an ideal situation: We cannot yet follow a clear breeding scheme ending with higher yield or better quality - but the potato offers a fair chance to achieve some progress in breeding via in vitro techniques today.

Table 1 Protoplast regeneration from economically important plants

Crop plants			Drug plants		
Asparagus officinalis		(5)	*Atropa belladonna*		(17)
Brassica napus	2n	(6)	*Datura innoxia*	2n, 1n	(18)
	1n	(7)	*Datura metel*	2n, 1n	(19)
Citrus sinensis		(8)	*Datura meteloides*	2n, 1n	(19)
Daucus carota		(9)	*Hyoscyamus muticus*		(20)
Lycopersicon esculentum		(10)			
Nicotiana tabacum	2n	(11)	Horticultural plants		
	1n	(12)			
Solanum tuberosum	2n	(13,14)	*Petunia hybrida*	2n	(21)
	1n	(15,16)		1n	(22)
			Petunia parodii		(23)

Remarks on MATERIAL AND METHODS

A first step towards the regeneration of potatoes from protoplasts was reported by Lorenzini (24) starting from tuber tissue; Upadhya (25) solved the problem of inducing in vitro proliferation of mesophyll protoplasts, Shepard and Totten (13) and Butenko et al. (14) regenerated the tetraploid potato to functional plants, and the system was completed by the regeneraion of haploid protoplasts by Binding et al. (15) and Melchers (16). In the line of regeneration experiments starting with varieties, Shepard and co-workers (26, 27) could demonstrate a striking variability in the regenerants which will be discussed in more detail later. Besides this use of protoplasts for an intracultivar improvement, simple protoplast regeneration might gain some importance as a phytosanitary technique or for a very rapid propagation of selected valuable genotypes. The real potential of protoplasts in potato improvement is offered by somatic cell fusion. As the potato is an autotetraploid with 48 chromosomes, fusion of normal protoplasts will result in octo-

ploids, which are of no practical value. Therefore, we concentrated our research on protoplasts isolated from dihaploid potatoes possessing 24 chromosomes which can be extracted from tetraploids fairly efficiently via a parthenogenetic procedure (28). Such dihaploids are of increasing importance in modern breeding programs (29). In our breeding program for instance, 80 % of this year's 120,000 seedlings have haploid ancestors. Within clones having 24 chromosomes one has to dintinguish between dihaploids from commercial varieties, dihaploids from primitive varieties and wild species. The regeneration of dihaploid *S. tuberosum* could be achieved (15,16) and now also the isolation of protoplasts from primitive varieties, wild species and hybrids (30,31), and even from monohaploids is possible. Such monohaploids (Fig. 2) were first described by Baerecke (cit. Frandsen, 32) and then produced in a wider range via parthenogenesis by Hermsen (33). We start from monohaploids which were produced via microspore culture within the anthers (34).

Conditioning of the plants before enzymatic treatment is a very critical step in potato protoplast isolation as reported by Shepard and Totten (13) and also by Grun and Chu (30). Shepard and Totten recommended a very controlled growth where the plants are fertilized before harvesting with mineral salt solutions according to Murashige and Skoog's medium (35) with a twofold increase in $CaCl_2$ and KH_2PO_4 concentrations. Plants were maintained in climate chambers at 18°C under a 12 h photoperiod at 4,000 lux. Grun and Chu (30) stored the excised leaves in low light (3,000 lux) or in darkness for 2-5 days at room temperature before enzyme digestion. For our protoplast isolations (15) we grow the donor material as shoot tip cultures. Such starting

Fig. 1. Potato plants in the field grown from tubers of protoplasts derived regenerants of the dihaploid clone H^2260

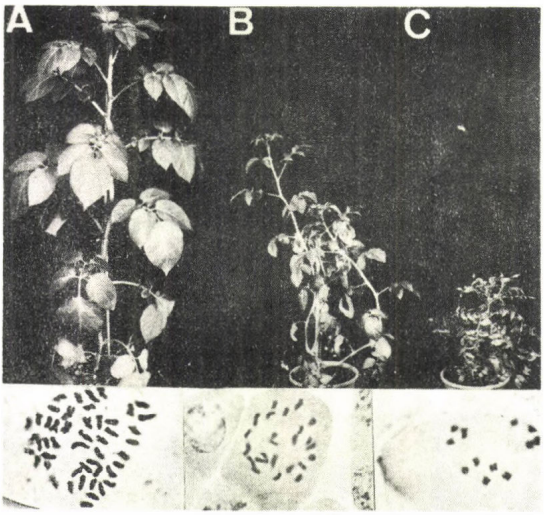

Fig. 2. Potatoes with different ploidy levels:
A - tetraploid with 48,
B - dihaploid with 24,
C - monohaploid with 12 chromosomes.

material has been proposed by Binding (36) for a very controlled isolation of *Nicotiana* protoplasts. It offers some striking advantages: The preculture of the plants can be optimized and consequently the physiological conditions are much more constant than in greenhouse grown plants. Furthermore, the sterilization of the leaflets can be omitted. For this procedure from dihaploid clones, first shoots are grown from meristems; from primitive varieties and wild species the cultures are started from seeds. The medium used for culture is that of Murashige and Skoog (35) supplemented with 0.5 mg/l 6-benzylaminopurine (BAP) and occasionally with 10 mg/l thiamine omitting then cysteine. For subsequent culture, segments (about 1 cm long) are plated on new medium and exactly 3 weeks after such a transfer the shoots can be harvested, chopped in total and incubated in a preincubation medium of 0.3 M mannitol (37). As, however, the establishment of such cultures for applied aims is rather time-consuming, we also use greenhouse grown material for fusion experiments. These latter plants are grown under 16 h light extended during the winter season by Osram HQI-T 1,000 W lamps with a light intensity at the growing points of the plants of 15,000 lux. The temperature is kept below 20°C and additionally the plants are grafted on tomato stocks.

The dihaploid clones we started our experiments with were selected according to three major points: 1. For these clones knowledge about how to handle them in tissue culture from callus (38) or microspore (34) regeneration experiments was already available. 2. The clones were either carrying qualities important for application like virus resistance, or they possess a special phenotype, the nodal band marker (39), facilitating an identification after fusion experiments. 3. A prag-

matic point, they were the best dihaploids available, according to vigour and quality.

For isolation of the protoplasts all authors use the single step method with an incubation in cellulase and pectinase together. We routinely incubate in a 0.2/0.2 % mixture for 4 h at 25°C on a roller and clarify the protoplast suspension by banding on 50 % Percoll solutions. Each group favours for regeneration a different medium: Shepard and Totten (13) composed their own medium which resembles that of Nitsch and Ohyama (12) in the composition of major salts, like the medium which Grun and Chu (30) used. Melchers (16) and we (15) induced first divisions in the medium developed by Kao and Michayluk (40) for which Binding et al. (15) even found a wider applicability. In common all groups add BAP as phytohormone. For the induction of morphogenesis we proved zeatin to be essential in a concentration of 0.3 mg/l (15, Fig. 3a); rooting started on phytohormone-free Murashige and Skoog's (35) medium. Besides tetraploid *S. tuberosum* varieties and clones (13,14), regeneration could be started in a spectrum of dihaploids: *S. chacoense*, *S. infundibuliforme*, *S. phureja*, *S. sparsipilum*, *S. tarijense*, and of the hybrid *S. phureja* x *S. chacoense* (14, 30, 31).

In the beginning we used for our protoplast fusion experiments the method developed by Keller and Melchers (41). As shown in Table 2, the protoplasts are fused at high Ca^{++} concentration and at high pH. The efficiency was further enhanced by addition of 4.5 % polyethylene glycol (PEG, 42). This is a very gentle method, allowing the survival of most protoplasts. By the method of Kao and Michayluk (43) with five times more PEG, the relative rate of fusion products is higher, but the total survival rate is lower. As no conditions are known at the moment to allow only the hybrids to grow, and consequently many plants have to be regenerated, we prefer now the method with high PEG.

For preselection we exploit the extreme vigour of calluses descending from fusion products (44). Especially for applied aims in breeding programs, hybrids which express the good combining ability of their parents (Fig. 3b) will be of predominant interest. Fusion products with decreased vitality are overlooked, but the chance that they deliver hybrid clones is rather limited.

Application of isoenzyme techniques will allow further identification of possible hybrid plants (44). Experiments which also use the mechanical isolation of fusion products (46) are under investigation.

In some fusion experiments - especially for testing the efficiency of our system in a non-pragmatic way - we adopted the method of complementation using chlorophyll deficient mutants (47) grown in sterile shoot tip culture on Murashige and Skoog's medium (35) supplemented with 1 % glucose. These mutants were produced by irradiating anthers before microspore culture with γ-rays (^{137}Cs, 48).

Table 2 Procedures used for fusion of dihaploid protoplasts of potato

	Method according to: Schieder (45) var. Keller & Melchers (41)	Gleba & Hoffmann (46) var. Kao & Michayluk (43)
a/ Fusion medium		
PEG	4.5 %	25 %
Ca^{++}	275 mM (as nitrate)	50 mM (as chloride)
pH	10	9
time	10 min	15 min
b/ Washing medium		
Ca^{++}	---	50 mM
pH	---	11
time	---	5 x 5 min

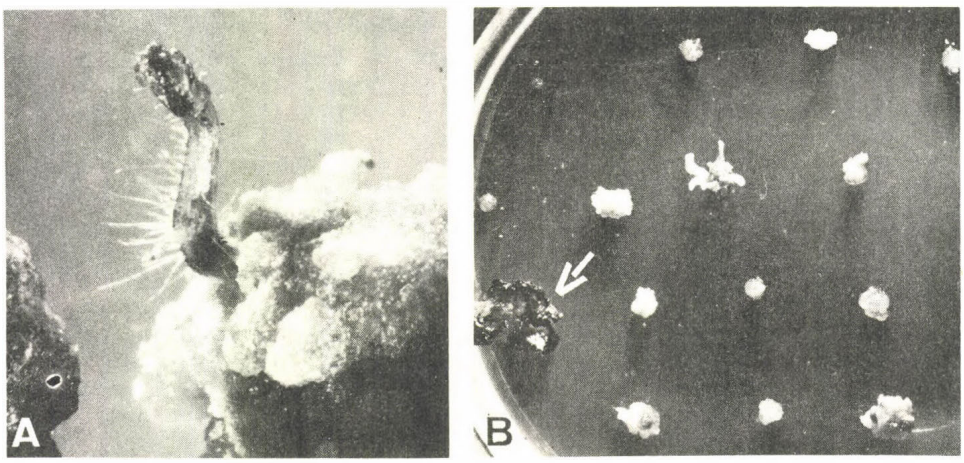

Fig. 3. Regeneration of dihaploid protoplasts. A/ Start of morphogenesis. B/ Calluses regenerated after fusion experiments; one of the calluses expresses increased vigour and in addition a different colour.

RESULTS AND DISCUSSION

The main aim of our pragmatic experiments is to improve potatoes. As already outlined we focused on the use of dihaploid protoplasts, fusion products of which will replace steps in classical breeding programs. Besides fusion of dihaploids there exist also some results promising the incorporation of tetraploid protoplasts into plant breeding. Shepard (27) and Butenko et al. (14) observed a wide range of variants after protoplast regeneration. Even after having discarded aberrants due to polyploidy or aneuploidy there still existed minor morphological aberrants expressing e.g. an altered level of resistance to *Alternaria solani*, to *Phytophthora infestans* and an altered pattern in plant geometry, tuber phase, earliness, depth of tuber set, amount of seeds and tuber shape (26, 27). These differences even have a parallel in nutritional requirements. Grun and Chu (30) and we (15, 31) found that plants rapidly regenerated from mesophyll protoplasts of shoot tip origin delivered homogeneous populations according to phenotypic characters. Except for some aberrations being polyploid, the population looked very uniform (Fig. 1), at least the variation did not differ from variations observed in clones grown from tubers or seeds under similar environmental conditions. Although there was a high rate of spontaneous doubling from 24 to 48 chromosomes, the rate of aneuploids did not exceed 4 %. Most aneuploids could be easily detected by their very dark green colour and deformed leaves. In protoplast derived clones isolated from suspension cultures, which we overtook from Melchers (16, 31), we found a wider range of phenotypic variability which corresponded to a high amount of aneuploids. On the other hand, Melchers found a useful mutation (49) during early protoplast regeneration experiments with tobacco (11): from the first tobacco plants regenerated from mesophyll protoplasts one plant turned out to have changed from day neutral into a short day type, producing many more leaves under our photoperiodic conditions. The genetic basis for this type of mutation remains to be clarified. Simply the number of chromosomes cannot be the reason, as this was checked carefully e.g. for the potato (27), but smaller aberrations from the caryotype are not easy to identify. As I was in favour of pragmatism up till now, I cannot be too critical in this case. I think, however, that the use of such undirected aberrants as a general intracultivar improvement will be highly questionable.

Besides this system probably starting at the single cell level and therefore simplifying the detection of spontaneous somatic mutants, protoplasts promise their highest potential in combination breeding.

First I would like to make a short excursion into normal breeding processes of the potato, where selection of parents according to the breeding aim, crossing and subsequent selection amongst the segregating F_1 progeny follow one after the other. Per year we produce 100,000 - 150,000 seedlings from which we select over a six-year period the 5 - 10 best. The chance to find those in a positive selection procedure is so small that one uses negative selection which means that all non-optimal

looking types are discarded, and only the rest are maintained. In other words, all good plants are thrown away and only the best ones are grown again. The number of seedlings which can be managed in selection is the bottle neck; any procedure possessing a higher efficiency allows reduction of the total amount of seedlings, or will deliver a greater number of superior plants. The potential of the classical technique is decreased with the increasing number of new breeding aims. An optimal combination of the available techniques for a mixed application of in vitro methods and classical approaches may overcome this difficulty.

Here we developed the following breeding scheme (31): From autotetraploid clones dihaploids are extracted via a parthenogenetic technique, the interspecific cross with *S. phureja* (28). Starting from normal highly hetezygous tetraploids, the dihaploids will still be heterozygous. To put combination breeding on a more predictable basis the dihaploids are made homozygous via a passage through the monohaploid level with subsequent doubling. Each step is accompanied by selection for the desired genotypes. The homozygous dihaploids are crossed sexually to yield a hybrid which combines several characters of the parents. From such hybrids protoplasts are isolated and fused with other selected hybrids to result in a completely heterozygous new tetraploid plant, in which characters of at least four starting lines are combined. The doubling of the dihaploids onto the tetraploid level during fusion is given into the bargain.

To avoid any misunderstanding, this does not mean that fusion might displace the classical techniques! First one has to select conventionally on the dihaploid level and only thereafter one can combine selected best material, but now avoiding the normal meiotic segregation.

Following this scheme, we are at the moment restricted to available genotypes, possessing tissue culture ability and/or some of the desired qualities. To begin with we concentrated our experiments on characters with qualitative inheritance. Such either - or reactions simplify the detection afterwards. The system has of course its higher potential in quantitatively inherited characters, like yield, quality and some resistances, e.g. against potato leaf roll virus (PLRV) and to the nematode *Globodera pallida*, both polygenically inherited. Via protoplast fusion, there is a fair chance to combine such polygenes of two dihaploids in a new tetraploid.

Besides interhybrid fusion amongst *S. tuberosum* clones, primitive varieties or wild species might also be incorporated. For instance from the species *S. brevidens*, not sexually compatible with *S. tuberosum*, genes for PLRV resistance might be extracted, or from *S. sparsipilum* the resistance to *G. pallida*. Up till now we only fused *S. tuberosum* protoplasts with a *S. phureja* clone carrying the nodal band marker. As the amylase isoenzyme patterns of *S. phureja* and of the dihaploids predominantly used as one fusion partner are significantly different, an identification of fusion products should be possible. During fusion with wild species, many undesired genes are also introduced into the *tuberosum* genome and it will take time to select against these characters. With some exceptions there will be a wider applicability of intraspecific, predominantly interhybrid fu-

sions at this particular time (50).

As shown in Figure 4, up till now we fused mainly protoplasts from the dihaploid clone H^2 258, which possesses field resistance to the polygenically inherited PLRV and in addition the major gene for field resistance to potato virus Y (PVY). This clone is grown aseptically as shoot tip culture. As fusion partner we prefer greenhouse grown clones carrying the dominant nodal band marker and the major gene for field resistance to potato virus X (PVX) and/or genes for high seed production. From this greenhouse grown material the majority of the protoplasts will not regenerate under our cultural conditions but its dominant marker should be detectable in fusion hybrids. To date we regenerated about 2,000 plants (Fig. 4). Selection of this material is under trial. As a preselection system we used the hybrid vigour and threw away 90 % small calluses not expressing strong vigour. In an aliquot of 40 calluses (from more than 1,000) we checked the ploidy level and found that the calluses growing rapidly revealed the tetraploid chromosome number.

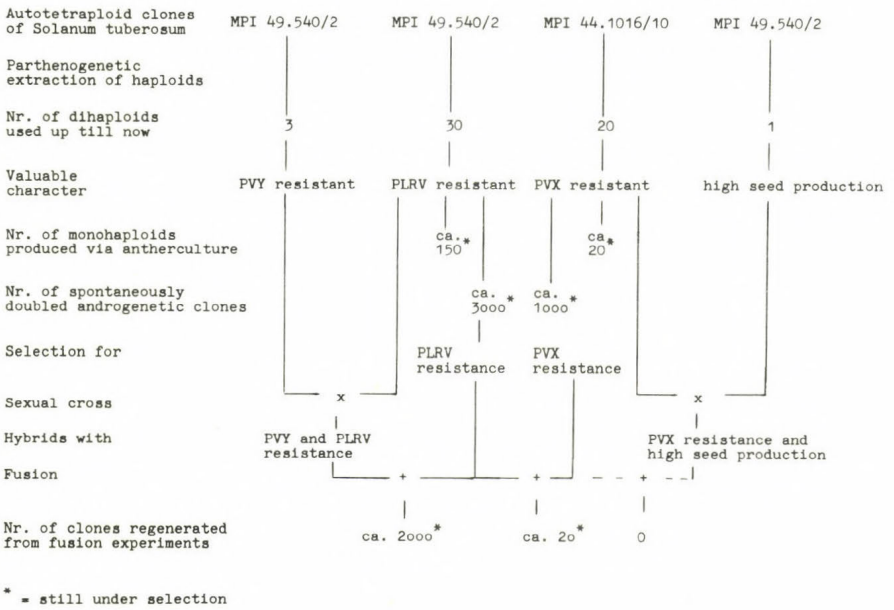

Fig. 4. Scheme for a combined application of classical and in vitro techniques in potato breeding, and the data of the clones under investigation at present.

The large calluses are induced to form shoots and are selected for the presence of the nodal band marker - up till now three plants are found with this marker. To make sure that it is not only this parent which grew in the presence of a regenerating second clone, now the isoenzyme pattern is checked to give a further hint. The final proof will be the field test which has to prove the presence of the valuable characters of both parents.

In parallel to this strict selection, we also follow a milder scheme. Besides the first preselection, which is necessary to reduce the number of regenerants to a manageable size, we grow all plants to maturity and will test them in the field in their tuber derived generation. Citing pragmatism again, it is not so important to prove that after the fusion experiment the offspring is really a somatic hybrid, than to demonstrate that the product is better than each of the parents and that the selection was more efficient compared to the classical approach.

CONCLUSION

Before summarizing, I would like to mention briefly progress with protoplasts in other applied work with economically important plants. First one has to refer to tobacco where besides the new day neutral line (49), Gleba (51), Belliard et al. (52), and Zelcer et al. (53) transferred via fusion the cytoplasmic male sterility (cms plastome). Also in *Petunia* cms cytoplasm transfer may facilitate hybrid seed production (54). In the drug plant *Datura* Schieder (55) was successful in increasing the alkaloid yield via fusion in new amphidiploid and consequently fertile hybrids. Several other approaches are discussed in detail in this volume. The very precise breeding aims in drug plants encourage the application of in vitro techniques as the economic risk is more calculable. The highest potential of in vitro techniques is offered when clear and not too complicated breeding aims are focused, like the transfer of just one character from one variety to another or the increase of just one component. I favour the small step approach, although spectacular fusions like potato with tomato (56) may gain an applied touch.

To summarize in a not too scientific way, I would state: Tissue culture still has the flavour of alchemy - for plant breeding one needs green fingers. As long as higher plant genetics and higher plant physiology cannot predict what happens after the combination of different genomes. I would recommend following pragmatic ways as a green-fingered alchemist.

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PROTOPLAST FUSION TO STUDY CYTOPLASMIC TRAITS IN *NICOTIANA*

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ABSTRACT

Kanamycin resistance and pigment deficiency of the *Nicotiana sylvestris* cell line, KR103, have been characterised as cytoplasmic traits after fusion with *Nicotiana knightiana* protoplasts. Pitfalls of such an analysis are discussed.

Plants regenerated from the somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana* contain only *N. knightiana* chloroplasts. This finding may be explained by nucleo-cytoplasmic incompatibility, or the choice of different cell types (tissue culture, and leaf mesophyll, protoplasts) for fusion.

The use of an isolated heterokaryon-nurse culture system to obtain clones from the *Nicotiana tabacum* + *Arabidopsis thaliana* heterokaryons is described.

INTRODUCTION

Sexual crosses between *Nicotiana* ssp. yield hybrids with only one type of chloroplasts and mitochondria since they are inherited uniparentally (maternally). By fusing protoplasts, mixed organelle populations can be obtained which makes possible novel approaches to the study of cytoplasmic organelles in flowering plants (1,2,3,4,5).

In this paper experiments are described in which we used protoplast fusion to characterise a *Nicotiana sylvestris* cell line (KR103), to study the fate of chloroplasts in *Nicotiana tabacum* + *Nicotiana knightiana* somatic hybrids, and to exploit uniparental streptomycin resistance in screening for somatic hybrids.

CHARACTERISATION OF KANAMYCIN RESISTANCE AND PIGMENT DEFICIENCY OF THE *N. SYLVESTRIS* CELL LINE, KR103

Kanamycin resistance. Plant regeneration from the kanamycin resistant line, KR103, is not possible (6), so nuclear or cytoplasmic location of the resistance factor could not be determined in sexual crosses. Location of the resistance factor was attempted by somatic genetic studies. Resistant *N. sylvestris* (KR103) protoplasts were fused with sensitive *N. knightiana* protoplasts and the somatic hybrids were selected (7). Subsequently, isolation of resistant and sensitive hybrid subclones was attempted since segregation is expected if the trait is cytoplasmic (1,2,3,4,5). Correlation between resistance and the presence of a *N. sylvestris* (KR103) organelle was to be confirmed by identifying chloroplasts and mitochondria in the subclones.

Initially, all hybrid calli were resistant to kanamycin whether (7) or not (8) kanamycin resistance was used for selection to isolate the hybrids. Kanamycin resistance of the KR103 line is therefore considered to be a dominant trait.

In order to allow time for the segregation of the organelles the hybrid cells were grown for many cell generations. Portions of the H1 and H2 hybrid calli (7) were subcultured several times on selective agar medium containing 50 ug/ml kanamycin sulphate or in kanamycin-free liquid medium. The sublines, that is cultures of the same line maintained independently during an 18 month period, were tested for resistance to kanamycin after three to five subcultures in absence of selection pressure (Table 1). Some of the lines are fully resistant while others are as sensitive as calli initiated from sensitive plants. Most of the lines exhibit intermediate levels of resistance. The values are distributed continuously between the two extremes.

In cultures of the sensitive and weakly resistant sublines the appearance of resistant sectors is frequent. Such a resistant segregant is the H2-61P1R2 callus line which was isolated from leaf callus of the sensitive H2-61P1 plant (Table 1). The ability to segregate resistant subclones was maintained therefore, through plant regeneration. The plants regenerated from the H1-42, H1-62 and H2-70 lines are all sensitive (Table 1).

At the time of fusion the KR103 cultures contained predominantly cells with 72 chromosomes (n=X=12), and low proportion with 96 chromosomes. Mesophyll cells of *N. knightiana* were prepared from diploid (2n=2X=24) plants. Plants regenerated from the somatic hybrid callus contain different and variable numbers of chromosomes. For example in root tips some mean values were: H1-23BK plant, 71 chromosomes (range: 54-84), H1-42SP2 plant, 60 chromosomes (range: 52-67), H1-62P1B plant, 63 chromosomes (range: 61-66). Since chromosome numbers are lower than the addition of the numbers in the parental cells the possibility that the loss of resistance is due to the selective elimination of the KR103 genome was considered. To test this hypothesis isoenzymes were studied in the sublines because this technique proved useful to detect chromosome (total genome) elimination in soybean + *N. glauca* somatic hybrids (9).

TABLE 1 Segregation for kanamycin resistance in the somatic hybrid cell lines of *N.sylvestris* (KR103) + *N.knightiana*

	Growth on kanamycin medium		Growth inhibition (%) by 50 ug/ml kanamycin
	0 ug/ml	50 ug/ml	
Parental lines			
N. sylvestris	987 ± 116	94 ± 11	10
N. knightiana	656 ± 46	28 ± 2	4
KR103-R	926 ± 111	991 ±150	107
KR103-S	1189 ± 125	46 ± 6	4
Hybrid clones			
H1-23	558 ± 83	32 ± 6	7
H1-62B	1138 ± 67	30 ± 5	3
H1-210K	1096 ± 167	12 ± 1	1
H2-61P1R2	1102 ± 112	1127 ± 76	102
H2-73	1156 ± 54	1013 ± 70	86
H2-S54	654 ± 92	289 ± 29	44
H2-S7	1143 ± 127	321 ± 95	26
H2-22Z	1244 ± 172	140 ±108	11
Callus from plants			
H1-42SP2	1259 ± 143	40 ± 4	3
H1-62P1B1	1219 ± 70	55 ± 12	5
H2-70P1A	828 ± 196	59 ± 12	7

Resistance was scored as the ability to grow (mg fresh wt. ± s.e.m.) on RMP medium containing 50 ug/ml kanamycin sulphate. Calli (initial wt. 20-25 mg) were grown in Petri dishes (9 pieces in a dish with a 10 cm diameter) containing 20 ml of medium. Cultures were incubated in darkness and weighted after 5 weeks. Culture procedures have been described in more detail previously (7).

Sensitive and resistant cell lines, and sensitive plants, maintained the hybrid patterns as indicated by studies of the esterase and alcohol dehydrogenase isoenzymes. Esterase isoenzymes from leaves are shown in Fig. 1. The renegenerates exhibit a combination of the morphological characteristics of the

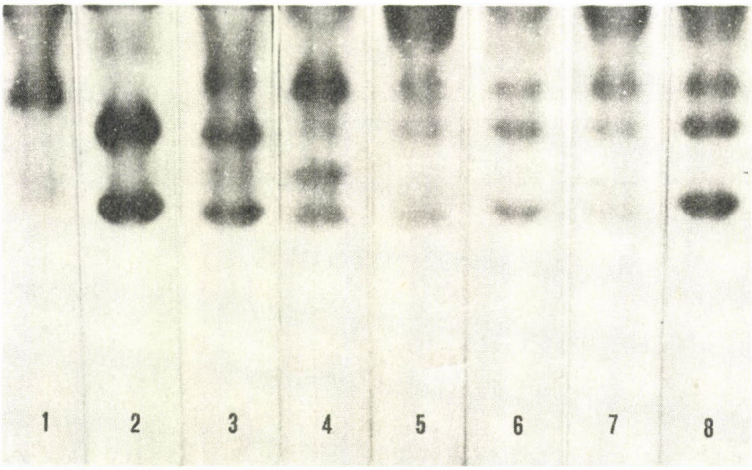

Fig. 1. Esterase isoenzymes in leaf extracts of *N. sylvestris* + *N. knightiana* somatic hybrid plants after separation by polyacrylamide gel electrophoresis. Samples (100 ul) were run from *N. sylvestris* 1, *N. knightiana* 2, mixed 1+2 3, H2-70P1B 4, H1-62P1B1 5, H1-42SP2 6, H1-62P1B3 7, and H1-42SP4 8. Methods as described by Maliga et al. (7).

parents, winged leaves of *N. sylvestris* and hairiness of *N. knightiana*. The chromosomes of the resistant parent therefore have not been completely eliminated. Loss of chromosomes which are not coding for the studied isoenzymes, however, cannot be excluded.

Dominance of kanamycin resistance in the somatic hybrids, sorting out of lines with different and continuously distributed levels of resistance and maintenance of some of the resistant determinants even in sensitive cells, indicates that kanamycin resistance being coded for by a large number of genetic determinants which may easily segregate during mitosis. Experiments from other laboratories (1,2,3,4,5) indicate that this behavior is predictable if the trait is coded by organellar DNA. On the other hand the variable chromosome numbers suggest that our results can also be explained by chromosome segregation in polyploid cells. Independent segregation of kanamycin resistance and parental nuclei in isolated heterokaryons (8), however, supports the cytoplasmic nature of kanamycin resistance.

The KR103 cells, from which the protoplasts were prepared for fusion, have been grown on non-selective medium. We realised during the course of these experiments that the KR103 line segregates sensitive cells (data shown in Table 1). It cannot be excluded that the protoplasts which were involved in fusion contained also some sensitive determinants. Studies on the identity of organelles therefore should not yield unequivocal results, and it will not be investigated. Instability is not a general characteristic of the kanamycin resistant lines since, e.g. the KR115 line, isolated at the same time (6) maintains full resistance.

Pigment deficiency. KR103 cells grow as white, unpigmented callus, even on "greening medium" (6) which may be due to nuclear or cytoplasmic mutations. In the absence of plant regeneration the choice between these alternatives, could be attempted by looking for mixed chloroplasts populations in somatic hybrid cells, an indication, that pigment deficiency is due to plastome mutation (classification of plastome mutations at the plant level is discussed by Hagemann in ref. no. 10.). Electronmicroscopic investigations revealed such cells, containing the proplastid-like structures of KR103 and normal chloroplasts from *N. knightiana* (7). As predicted, green leaves of the H1 (H1--62P1B1) and H2 (H2-70P1B) regenerates contained only *N. knightiana* chloroplasts (Fig. 2). Appearance of white sectors on the leaves of some of the somatic hybrid plants indicates that some KR103 plastids were maintained during plant regeneration.

Interpretation of our data may be more complicated if we suppose that, in this species combination, the type of hybrid variegation exists, which has been described in *Oenothera* (12). Sexual hybrids, or somatic hybrids with normal *N. sylvestris* plastids are not available to exclude this possibility.

CHLOROPLAST SEGREGATION IN THE *N. TABACUM* + *N.KNIGHTIANA* SOMATIC HYBRIDS

Somatic, unlike sexual, hybrids may contain chloroplasts of either parent, that is, there is biparental transmission of chloroplasts (1,2,3,4,5). Sexual hybrids of *N. tabacum* and *N. knightiana* could be obtained only by using *N. knightiana* as a female parent (13). Production of hybrids with the *N. tabacum* chloroplasts was therefore attempted by somatic hybridisation.

Experiments of Chen et al. (2) indicate that in the somatic hybrid cells a mixed chloroplast population is maintained for a relatively long period of time, and regeneration of plants with different chloroplasts is possible from the same hybrid clone. A number (altogether 28) of shoots was therefore regenerated from the available 3 somatic hybrid calli, grown into plants, and the chloroplasts were identified by the species

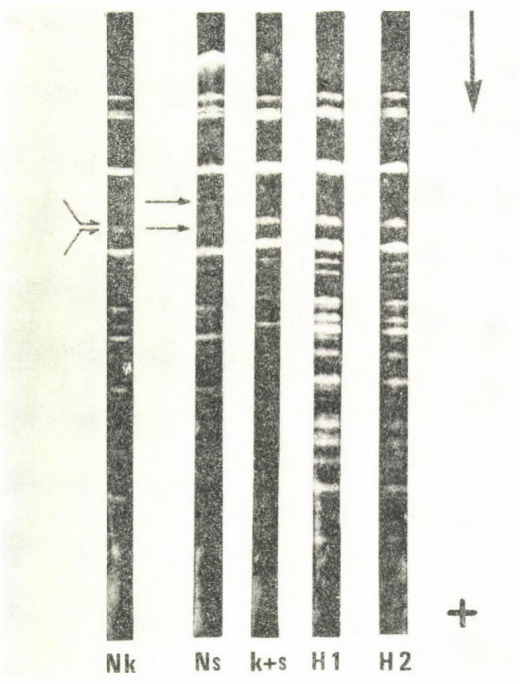

Fig. 2 Identification of chloroplasts in the leaves of the *N. sylvestris* + *N. knightiana* somatic hybrids by the restriction pattern of chloroplast DNA. Patterns of *N. sylvestris* (*Ns*), *N. knightiana* (*Nk*), and H1 (H1-62P1B1) and an H2 (H2-70P1B) regenerates are shown. Methods as described by Atchison et al. (11).

specific restriction patterns of the chloroplast DNA's.

All the regenerates contained exclusively *N. knightiana* chloroplasts, that is the somatic hybrids are of the same type as those which can be produced by sexual crosses. This may be explained by the incompatibility of *N. tabacum* chloroplasts with the hybrid nucleus. To test this hypothesis, production of such hybrids was attempted by *in vitro* pollination. This experiment is inconclusive so far since the six plants obtained after pollinating *N. tabacum* with *N. knightiana in vitro* turned out to be parthenogenetic *N. tabacum*. Alternatively, our results may be explained by the successful competition of chloroplasts derived from the leaf mesophyll protoplasts of *N. knightiana* with the smaller number of proplastids of the *N. tabacum* protoplast isolated from a cultured cell.

USE OF THE ISOLATED HETEROKARYON - NURSE CULTURE SYSTEM TO SELECT CLONES DERIVED FROM *N. TABACUM* + *ARABIDOPSIS THALIANA* HETEROKARYONS

After fusing tissue culture protoplasts and leaf mesophyll protoplasts, heterokaryons of *N. sylvestris* and *N. knightiana* could be identified by their characteristic morphology. Heterokaryons were isolated by micropipette and transferred into an albino nurse culture. One-fifth of the green calli (4 out of 25), found among the white nursing calli were shown to be somatic hybrids (8). Similar data were obtained by Gleba and Berlin (14) in a single cell system.

We used our isolated heterokaryon-nurse culture system to study the viability of the heterokaryons of the distant species *N. tabacum* + *Arabidopsis thaliana*, hoping to recover a few somatic hybrids among the heterokaryon-derived clones. Protoplasts, prepared from leaf mesophyll cells of the uniparental streptomycin resistant tobacco mutant, SR1 (15), were fused with protoplasts, prepared from a suspension culture of *A. thaliana* race Columbia. Isolation and culture of *Arabidopsis* protoplasts (16) and the method of fusion induction (7) have been described previously. Heterokaryons were isolated after 72 hours and cultured in a streptomycin sensitive tobacco nurse in liquid medium under conditions which favoured callus formation from tobacco protoplasts (K3 medium, ref 17.). Small calli obtained after three weeks time were plated into selective RMB medium (7) containing 300 ug/ml streptomycin sulphate. In this medium sensitive nursing cells form white calli. In cultures into which 335 heterokaryons were mixed, 59 resistant green calli were recovered. These calli were further grown on drug-free plant regeneration medium (RMO, ref. 15). In a similar size sensitive tobacco population no green calli were found. Data on the esterase and alcohol dehydrogenase isoenzymes indicate that the 12 lines studied so far contain no genetic material from *Arabidopsis thaliana*.

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MITOCHONDRIAL HYBRIDIZATION BY FUSION OF TOBACCO PROTOPLASTS WITH DIFFERENT SPECIES CYTOPLASMS *(DEBNEYI* AND *TABACUM)*

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INTRODUCTION

The protoplast fusion technique offers an experimental approach to the genetic study of nucleo-cytoplasmic interactions in the male sterile system. The process is unique in that it provides a means of combining the total genetic information (nuclear and cytoplasmic) of two cells. We describe a mixture of two genetically different cytoplasms as "hybrid cytoplasm". Plants derived from such a cell should inherit cytoplasmically determined characters from one or both of the parental strains. We chose to study regenerated plants from protoplast fusion of normal *tabacum* protoplasts and protoplasts of *N. tabacum* with *debneyi* cytoplasm. These cytoplasmic male sterile plants possess flowers with ribbonned petals, stamens with rolled filaments ending in stigmates. These morphological characters are our genetic markers of *debneyi* cytoplasm. Protoplast fusion between the two types leads to the formation of plants containing hybrid cytoplasm.

MATERIALS AND METHODS

Plant material. Protoplasts are isolated from three varieties of *Nicotiana tabacum* (2n=48). The varieties Samsun (Sf) and Xanthi (Xf) have petiolated leaves (a mendelian character) and normal flowers. Techne (Ts), the third variety, has been obtained by Tsikov et al. (1) from the interspecific cross: female *N. debneyi* x male *N. tabacum*, followed by backcrosses with *N. tabacum* as the male parent. These Ts plants possess the *tabacum* nucleus in *debney* cytoplasm, and are characterized by sessile leaves and abnormal flowers with male sterility. Therefore, the shape of leaves and the shape of flowers provide nuclear and cytoplasmic markers, respectively. Ts. Xf and Ts. Sf sexual hybrids have distinctive leaves with wings on each side of the petiole and are male sterile like the Ts parent.

Isolation and fusion of protoplasts. Leaf protoplasts are isolated according to Chupeau et al. (2) by using a mixed enzyme treatment. The peeled leaves are put on the surface of maceration solution containing 0.2 % cellulase R10, 0.1 % pectinase

R10, 11 % mannitol, 6 mM $CaCl_2$, 0.7 mM Na_2HPO_4 (pH 5.5) for 16 h at 20°C.

Fusions are achieved between protoplasts from one of the two fertile varieties (Sf or Xf) and protoplasts from the cytoplasmic male sterile variety (Ts). In the first experiments, Sf protoplasts were used but these were replaced by Xf protoplasts because plantlet regeneration, *in vitro*, from Sf protoplasts could not be obtained in our experimental conditions. Protoplasts are induced to fuse according to Kao and Michayluk (3). Each experiment is constituted by six assays in order to estimate the fusion ratio induced between Ts and Xf (or Sf) protoplasts, by polyethylene glycol 1500 and to rule out artifacts induced by *in vitro* culture:

1	M_p :	Ts + Xf (or Ts + Sf) with PEG 1500	
2	M_o :	Ts + Xf (or Ts + Sf) without PEG	)
3	Ts_o :	Ts alone without PEG	)
4	Xf_o :	Xf alone without PEG	) controls
5	Ts_p :	Ts alone with PEG	)
6	Xf_p :	Xf alone with PEG	)

Protoplast culture and plant regeneration. After the fusion treatment, protoplasts are cultured in the following medium: Murashige and Skoog macro-nutrients diluted at half; Heller micro-nutrients; Morel vitamins ; 37.3 mg/l Na_2EDTA plus 27.8 mg/l $FeSO_4.7H_2O$; 20 g/l sucrose; 100 g/l glucose; 750 mg/l bovine serum albumin fraction V; 1 mg/l naphtalene acetic acid (NAA) and 0.2 mg/l 6-benzyl-adenine, pH 5.8. The cell density of the culture is about 50,000 protoplasts per ml of liquid medium. After three weeks calli are plated on solid medium with 20 g/l sucrose, 1 mg/l 6-benzyl-adenine,no glucose and no NAA. Buds are formed in about 2-3 weeks and are transferred for root initiation on solid medium without any growth substances. In these experiments we have only transferred the first bud formed from each callus. So we have kept only one regenerated plant from each protoplast.

Isolation of chloroplastic (cp) DNA. The cp-DNAs are extracted as described be Vedel et al. (4). Chloroplasts prepared from deribbed leaves are treated by DNase to remove nuclear DNA. The DNase-treated chloroplast pellet is lysed by sarcosyl-pronase treatment and the lysate centrifuged on a two-step CsCl-ethidium bromide gradient. The cp-DNA is recovered as the fluorescent band and pelleted by ultracentrifugation.

EcoRI cleavage and electrophoresis of the cp-DNA restriction fragments. EcoRI restriction enzyme is prepared according to Thomas and Davis (5). The purified cp-DNA is exhaustively digested by EcoRI enzyme and the specific fragments are separated by electrophoresis on 0.7 % agarose gel.

Isolation of mitochondrial (mt) DNA. Green leaves are used because etiolated material cannot be obtained with tobacco. DNase-treated organelles are purified by centrifugation on two-layer gradient of 30 and 60 % sucrose. mt DNA is extracted as described for cp-DNA and then purified by linear CsCl-ethidium bromide gradient centrifugation, for 2 days at 105,000 g, in place of the two-step CsCl-ethidium bromide gradient. This last modifi-

cation leads to cleaner mt DNA bands.

Sal 1 cleavage and electrophoresis of mt DNA restriction fragments. Sal 1 enzyme is prepared according to Dr. Roberts (unpublished). Experimental conditions for mt DNA digestion and agarose gel electrophoresis are as previously described for cp-DNA.

RESULTS

Phenotypic distribution of the regenerated plants. The regenerated plants are distinguished first according to the shape of leaves, allowing us to determine the variety (Techne or Xanthi) or the nuclear somatic hybrids, then, according to the shape of flowers and the male fertility or sterility. As we have no early screening in our experimental system, we have regenerated about 2,500 plants. First, plants regenerated from control assays appear always identical to the initial plants, in respect to the leaf and flower shapes. These controls indicate that the *in vitro* culture as well as the PEG treatment do not induce phenotypical modifications regarding only the markers retained. Second, plants regenerated from the mixture of Ts and Xf protoplasts after PEG treatment fall into 3 classes: (1) 654 are identical to one or the other parent, in respect of both leaf and flower morphologies; (2) 213 possess leaves identical to the Ts parent and flowers intermediate between the two parental ones. These plants exhibit the characteristic chromosome number of *N. tabacum* (2n=48) and are named Ti. 12 plants have the petiolated leaves of the Xf parent but bear flowers intermediate between those of the two parents; they are named Xi; (3) 57 present modified flowers like Ti and Xi but also intermediate leaves (petiolated with wings). They are called Hi. These regenerated plants possess various chromosome numbers from 50 to 106. Some of them have 96 chromosomes. In a previous experiment, plants regenerated from the mixture of Ts and Sf protoplasts after PEG treatment fall into the above 3 classes in the following way: 451 Ts, 40 Ti and 2 Hi. (But we never obtained the Sf parent or Si types with our experimental conditions).

Floral phenotypic distributions of Ti and Xi plants and of their progenies. The shape of the flowers from Ti and Xi plants appears modified in different ways involving corolla, stamen filament and anther. The new phenotypes present a continuous variation between the sterile and fertile types. According to the good correlation found between the respective modifications of the flower morphology, the 253 Ti plants (213 from Ts + Xf and 40 from Ts + Sf fusion experiments) sort out into 4 classes and are randomly distributed between them. Plants belonging to class I are characterized by flowers producing pollen. However, their fertility is always lower than that of the normal parent and selfing requires "manual intervention" because of the reduced length of the stamens. Plants from classes II, III and IV are always male sterile. The major-

ity of the Ti plants bear only one kind of flower; yet, about 10 % of the Ti plants bear two or three flower types, each located on different stems. Only 12 Xi plants have been obtained. They exhibit the same variability of the floral phenotypes as the Ti plants. In the extreme cases, we get the transfer of the male sterility from one variety to another, or the restoration of fertility, since we obtain, by protoplast fusion, the transfers: Ts → Tf - and Xf → Xs. We have to verify if this transfer is definitive.

Verification of the stability and the maternal inheritance of new floral phenotypes of Ti, Xi and Hi plants, through vegetative multiplication and sexual generations. First we have kept the regenerated Ti, Xi and Hi plants for two or three years, in the greenhouse and have cut the principal shoot several times. We have also planted slips from them. We have observed the stability of each type of intermediate flower in these conditions. The offsprings of the following crosses have been studied in field and in greenhouse.

- Ti . Tf, Ti . Tf^2, Ti . Tf^3, Ti . Tf^4 and Ti . Xf.
- Tf . Ti and selfing for the Ti plants from class I.
- Xi . Xf, Xi . Xf^2, Xi . Xf^3, Xi . Xf^4 and Xi . Tf.

All the progenies appear phenotypically homogeneous and identical to the mother plant . The same results are obtained with the first, second, third and fourth generations. Each progeny includes 75 plants and the total analysis involves about 25,000 plants. These observations lead to important conclusions: (1) the modified floral characters are always cytoplasmically inherited; (2) the final stability of the new floral phenotypes obtained from protoplast fusion is demonstrated. Consequently, we have proposed the following hypothesis: - the Xi and Ti plants are cytoplasmic hybrids with only one parent nucleus: they are cybrids. - the Hi plants are both nuclear and cytoplasmic somatic hybrids. We have undertaken the analysis of the cytoplasmic DNAs of the first progenies of some Ti and Xi cybrids to establish the level of hybridization after the mixing of two species cytoplasms. We have used the specific cleavage of circular DNA molecules by restriction endonucleases.

Analysis of chloroplast DNA of some Ti and Xi cybrids with EcoRI enzyme. We have seen that the *tabacum* and *debneyi* cp DNAs have distinct restriction patterns. So, cp DNA, when cleaved by EcoRI enzyme, appears as a very useful genetic marker to distinguish *debneyi* and *tabacum* cytoplasms. Cp DNA of *N. tabacum* var. Samsun is identical to cp DNA of *N. tabacum* var Xanthi. We have chosen, for our study, homogeneous cybrids, bearing only one type of intermediate flower. Furthermore, the nine cybrids (6 Ti and 3 Xi) represent the total variability of the new phenotypes obtained by cytoplasmic hybridization.
It is worth noting that each cybrid contains only one or the other of the two parent cp DNAs:neither mixture norcombination have been observed on the electrophoretic patterns. According to the technique used, a ten per cent mixture of the two cp DNAs would have been detected. The initial mixture of the two

Table 1 - Relationship between floral phenotype and type of cp DNA

Plants	Classes	Male organ	cp DNA
parents			
Ts	-	sterile	*debneyi* D
Xf or Sf	-	fertile	*tabacum* T
T_{161}	I	fertile	T*
T_{47}	I	fertile	D
T_{27}	I	fertile	D
T_{1}	II	sterile	D
T_{75}	III	sterile	D
T_{13}	IV	sterile	D
X_{1}	IV	sterile	D*
X_{7}	IV	sterile	D*
X_{6}	II	sterile	T

*These plants represent the change of sterile Techne into fertile Techne and of fertile Xanthi into sterile Xanthi.

cytoplasms is thus followed by the elimination of one of the two parent cp DNA molecules. Table 1 also presents a comparison of the phenotypic marker of cytoplasm with the cp DNA for a given class of plants. It is clear that the flower morphology and the male sterility or male fertility could be changed whereas the cp DNA remained unmodified. This finding suggests that cp DNA is probably not involved or is not involved alone in cytoplasmic male sterility of tobacco. However, it is noticeable that the greatest modifications, represented respectively by passages from sterile Techne to fertile Techne and from fertile Xanthi to sterile Xanthi (see asterisks on Table 1), are correlated with cp DNA changes.

Analysis of mitochondrial DNAs. With F. Vedel, we have adapted the method of Kolodner et al. (6). Sal 1 digestion is used instead of EcoRI, because preliminary results have shown the EcoRI mt DNA restriction fragments to be too numerous.The mt DNAs from Ts parent (*debneyi* cytoplasm) and from Xf parent (*tabacum* cytoplasm) lead to two distinct Sal 1 restriction patterns. As shown by Table 2, Ts and Xf mt DNA restriction patterns exhibit 38 and 31 bands, respectively. Among these, 20 appear common to *debneyi* and *tabacum* mt DNAs, 18 are specific to Ts (*debney*) mt DNA, and 11 are specific to Xf (*tabacum*) mt DNA. Consequently, the pattern corresponding to a mixture of the two parent mt DNAs is constituted by 49 bands. We find that the three following varieties of *N. tabacum* used in our experiments, Xf, Sf and Tf (fertile Techne variety with the *tabacum* cytoplasm) have identical mt DNAs. Each of the nine cybrids contains a unique mt DNA quite distinguishable from both parent mt DNAs, and from their mixture. However, the cybrid patterns always contain mt DNA

fragments belonging to both parents and further, in all but one, new bands appear. The number of bands on the cybrid patterns varies from 35 to 41.

Table 2 - Sal 1 digests from mt DNAs

Plants		Classes (fertility or sterility)	bands				
			Common	*debneyi*	*tabacum*	New	Total
Xf, Sf, Tf		Fertile : F	20	-	11	-	31
Ts, Tsp		Sterile : S	20	18	-	-	38
Ti	T_{161}	I (F)	20	8	7	3	38
	T_{47}	I (F)	18	9	7	1	35
	T_{27}	I (F)	19	12	7	1	39
	T_{1}	II (S)	17	12	5	4	38
	T_{75}	III (S)	17	15	3	2	37
	T_{13}	IV (S)	20	18	1	-	39
Xi	X_{6}	II (S)	20	16	3	1	40
	X_{1}	IV (S)	19	17	3	2	41
	X_{7}	IV (S)	20	17	1	1	39

Xf, Sf, Tf: varieties with *tabacum* cytoplasm, normal and fertile flowers.

Ts : variety with *debneyi* cytoplasm, abnormal and male sterile flowers.

Tsp : *control plant:* plant regenerated from Tsp assay with abnormal and male sterile flowers identical to Ts parent ones.

Table 2 also reports the distribution of the specific parent bands and of the common ones, for each cybrid mt DNA. A correlation clearly appears between the flower shape and male sterility or fertility and the ratio of specific *debneyi* and *tabacum* bands. That is, an increase in the degree of sterility through the different phenotypical classes of the cybrids corresponds to an increase in the number of the *debneyi* specific fragments.

DISCUSSION

The fate of the mt DNA after the initial fusion of *debneyi* and *tabacum* cytoplasms is strikingly different from that of the cp DNA, in our cytoplasmic hybrid plants. Indeed, the present results, with the previous observations concerning the maternal inheritance and the stability of the floral markers provides evidence that protoplast fusion leads to the formation of new cytoplasms. The mt DNA plays an important role in the nucleo--cytoplasmic interaction determining floral morphology and male

sterility in tobacco. Related to the field of the nucleo-cytoplasmic interaction, the production of intermediate flower types has been reported before both by Tsikov and by Sand (7) from the backcrosses following the interspecific hybridization between *N. debneyi* and *N. tabacum* and they have demonstrated the nuclear determination of their intermediate flower types. However, according to our results, flower morphology and male sterility in tobacco are actually controlled by nucleo-mitochondrial interaction. The presence of new bands on the cybrid mt DNA restriction patterns is the physical proof of mitochondrial recombination in somatic hybrid cells. In animals, somatic cell fusion has been emphasized as a useful tool for mitochondrial genetics. In 1974, Dawid et al. (8) demonstrated mitochondrial recombination in hybrid cells derived from fusion of human cells and mouse or rat cells. In higher plants, advantages of somatic cell hybridization in studying "non-chromosomic" inheritance were first suggested by Gleba (9). Unfortunately our knowledge of mitochondrial genetics in higher plants is still limited, because of scarce biochemical and genetical markers (namely resistance ones). Moreover, it appears from recent data (10) that the organization of the mitochondrial genome of higher plants is much more complex than in animals. In this way, our results emphasize the potentially important applications of the protoplast fusion technique, namely for studying mitochondrial genetics and for increasing cytoplasmic variability, in higher plants. Furthermore, we have been able to transfer male sterility from one variety to another by protoplast fusion, indicating the fruitful aspect of this methodology.

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BIOCHEMICAL AND GENETIC ANALYSIS OF PLANTS DERIVED FROM THE FUSION OF X-IRRADIATED MALE STERILE *NICOTIANA TABACUM* PROTOPLASTS AND *N. SYLVESTRIS* PROTOPLASTS

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ABSTRACT

Recently we have reported on fusion between X-irradiated protoplasts of *line 92* (a cytoplasmic male sterile (CMS)) *N. tabacum* and protoplasts of *N. sylvestris* (1). Among the regenerated plants the majority (Type A) had shoot, leaf and perianth morphologies of *N. sylvestris* but their anthers resembled those of the CMS parent and in fact were male sterile. Some plants (Type B-1) were male sterile but their leaf, shoot and perianth morphologies were similar to the sexual F_1 hybrid *(N. sylvestris* x *N. tabacum)*. Other plants (Type B-2) had the shoot, leaf and flower morphologies of *N. sylvestris* but they were partially male sterile. All hybrid plants (total of 26) were pollinated with either *N. sylvestris* or *N. tabacum*. Progenies as well as androgenic plants were studied in respect to shoot and flower morphologies. Seedlings were germinated and analysed for tentoxin sensitivity. The results indicated that Type A plants consisted of *N. sylvestris* nuclei plus the cytoplasm of the CMS parent, that Type B-1 plants consisted of nuclei and cytoplasm of the CMS parent plus nuclear material of *N. sylvestris* and that Type B-2 plants consisted of nuclei and chloroplasts of *N. sylvestris* but possibly the mitochondria were contributed by the CMS parent.

In recent years a considerable number of somatic hybrid plants, resulting from protoplast fusion, were reported (see 2). Only in few of these reports the sexual progenies of the somatic hybrids were analysed (3-8). We previously reported (1) on the transfer of cytoplasmic male sterility from *line 92 (N. tabacum* nuclei with alien cytoplasm) to *N. sylvestris* by protoplast fusion. The somatic hybrid plants obtained were divided into three groups: Type A, Type B-1 and Type B-2, according to plant morphology and anther sterility. We now report on the androgenic and sexual progenies of these types and also provide information on a chloroplast inherited character: tentoxin sensitivity.

Shoot and flower morphologies of the original somatic hybrid plants of Type A and Type B-2 were identical with *N. sylvestris* and therefore it was assumed that they contain only *N. sylvestris* genome (1). This assumption was confirmed when Type A and Type B-2 plants were pollinated with either *N. sylvestris* or *N. tabacum* and the progenies' morphology was checked (Table 1).

TABLE 1 F_1 progeny of somatic hybrid plants pollinated with either *N. sylvestris* or *N. tabacum* cv. Xanthi

Female Parent and chromosome number	Male Parent	
	N. sylvestris	*N. tabacum* (cv. Xanthi)
Type A (2n=24)	Good seed setting; good germination. Shoot and floral morphologies identical to type A 2n=24	-
(2n=48)	-	Good seed setting; good germination. Shoot and floral morphologies like that of F_1 (*N. sylvestris* x *N. tabacum*). Anthers tapered and sterile. 2n=48
Type B-1 (2n=48-80)	-	Very few seeds. Shoot and floral morphologies variable. Stamens stigmatoid/petaloid or with tapered anthers. Sterile. 2n=48-72
Type B-2 (2n=48)	-	Good seed setting. Good germination. Shoot and floral morphologies like F_1 (*N. sylvestris* x *N. tabacum*). Pollen fertile but self sterile. 2n=48

Several plants of each somatic hybrid type were used as female parents and 20 or more progeny plants of each sexual cross were characterized.

The progenies of each of these crosses were homogeneous. The original somatic hybrid plants of Type B-1 showed intermediate shoot and flower morphologies similar to the sexual F_1 hybrid *N. sylvestris* x *N. tabacum* and their progeny displayed hetero-

geneity, some were more like *N. sylvestris* and some were more like *N. tabacum*. These results were consistent with the suggestion that Type B-1 contained both *N. sylvestris* and *N. tabacum* genomes, or part of them.

Due to the relative late abortion of pollen in CMS *Nicotiana* (see 9) it was possible to obtain androgenic plants from male sterile plants (Table 2). The homogeneous morphology of the

TABLE 2 Androgenic progeny of somatic hybrid plants

Source of anthers	Total number of anthers cultured	Number of plants obtained		Structure of stamens/ /male fertility
		with reduced chromosome number	with non-reduced chromosome number	
Type A (10 plants)	561	18	5	stigmatoid/ sterile
Type B-1 (2 plants)	121	0	0	-
Type B-2 (2 plants)	82	1	0	normal/fertile

Flower buds (1.5-2.0 cm long) were removed from tested plants and anthers were cultured on solidified Nitsch medium (10) containing 0.25 % charcoal.

androgenic plants obtained by anther culture of Type A plants is consistent with the above mentioned assumption that Type A plants contain only *N. sylvestris* genome. On the other hand, all these 23 plants contained stigmatoid stamens and were male sterile suggesting that their cytoplasm was contributed by the CMS parent.

Unfortunately, we did not obtain androgenic plants from Type B-1, where great variation was expected if indeed they are composed of both *N. sylvestris* and *N. tabacum* genomes or part of them.

Anther culture of Type B-2 plants yielded only one plant which was morphologically similar to *N. sylvestris* and it also had normal, fertile anthers in contrast to the original Type B-2 plants which were self incompatible.

The cytoplasms of the original "parental" protoplasts differed in respect to several characters. One of these is sensitivity to tentoxin. It was shown (11) that some of the *Nicotiana* species are sensitive to tentoxin (a cyclic tetrapeptide produced by *Alternaria tenuis*) while others are insensitive.

It was also shown (12) that the reaction to tentoxin is cytoplasmic inherited and in fact it was shown that tentoxin binds specifically to chloroplast Coupling Factor no. 1 (13). *N. tabacum* as well as *N. sylvestris*, which is considered to be its maternal ancestor, are both insensitive to tentoxin, while *line 92* (CMS), which contains a non-*tabacum* plasmon, is sensitive.

All 26 original somatic hybrid plants were included in tentoxin sensitivity tests (Table 3). The hybrid plants were the mater-

TABLE 3 Tentoxin sensitivity test

Plant type	Tentoxin sensitivity	
	sensitive	insensitive
N. sylvestris		+
line 92	+	
Type A	+	
Type B-1	+	
Type B-2		+

Seeds obtained from all 26 somatic hybrids (19 Type A, 3 Type B-1 and 4 Type B-2) were placed on 1 cm filter paper discs in 0.3 ml water (control) or 0.3 ml water containing 20 ug/ml tentoxin (11). Seedlings which greened in tentoxin were considered insensitive while those which did not green were considered sensitive.

nal parents and either *N. sylvestris* or *N. tabacum* were used as paternal parents.The test was performed on the seeds derived from these crosses. As was shown before (12, 14) the reaction of the seedlings to tentoxin was irrespective of the pollinating parent and, therefore, represented the maternally contributed cytoplasm. All Type A and Type B-1 seedlings were sensitive to tentoxin while Type B-2 seedlings were all insensitive (Table 3). The results are consistent with the assumption that Type A and Type B-1 plants contain *line 92* plasmons while Type B-2 plants contain *N. sylvestris* plasmon. The two "parental" protoplasts differed in respect to two additional chloroplastic characters: Isoelectric focusing of the large subunit of RuBPCase and cp DNA restriction pattern (15). The results obtained with these two markers were consistent with those obtained for tentoxin.

From the progeny analysis of the somatic hybrids in respect to both nuclear and cytoplasmic markers the following conclusion can be drawn: 1. Type A plants contain *N.sylvestris* nuclear genome but *line 92* chloroplasts, thus representing

a new nuclear-cytoplasm combination. They are actually cybrids and not hybrids. 2. Type B-1 plants contain both *N. sylvestris* and *line 92* genomes (or part of them) but only *line 92* chloroplasts. Therefore they are true hybrids but their plasmon is of *line 92* only. 3. The fact that Type A and Type B-1 plants contain only one type of chloroplasts confirm previous observations (3, 5, 6) indicating that when fusion involves two different populations of chloroplasts there is a rapid segregation to either one or the other. 4. Type B-2 plants contain *N. sylvestris* genome and *N. sylvestris* chloroplasts but they differ from normal *N. sylvestris* in their partial sterility. Although we do not have direct evidence we propose that Type B-2 plants resulted from a fusion event in which *N. sylvestris* contributed both nucleus and chloroplasts and *line 92* contributed some other cytoplasmic entities e.g. mitochondria, resulting in partial sterility.

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SOMATIC HYBRIDIZATION FOLLOWING PROTOPLAST FUSION AS A TOOL FOR THE GENETIC ANALYSIS OF DEVELOPMENT IN THE MOSS *PHYSCOMITRELLA PATENS*

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INTRODUCTION

The life-cycle of the moss, *Physcomitrella patens*, which has been described in detail (1-3), is predominantly haploid and can be completed under defined culture conditions. Spore germination, which takes 1-3 days, is followed by growth of branching filaments (primary chloronemata). Chloronemal cells contain many chloroplasts and intercellular walls are perpendicular to the axis of the filament. 4-6 days later a second type of tissue develops; it consists of long peripheral filaments (caulonemata) radiating from the central chloronemata, growing perpendicular to the direction of incident light (4) and composed of cells which are longer and contain fewer chloroplasts than chloronemal cells. Intercellular walls are oblique and older cells contain red-brown pigment. Caulonemal cells divide to give rise to side-branches most of which develop into secondary chloronemata, a tissue which appears to be morphologically identical to primary chloronemata, although responding differently to varying intensities of unidirectional light (4). The chloronemata and caulonemata together constitute the protonema. A few caulonemal side branches develop into buds, the first buds being initiated about 10 days after germination, and each developing into a gametophore with a stem, leaves and rhizoids. Thus caulonemata is the main adventitious tissue, and chloronemata and gametophores are the main photosynthetic tissues. The formation of sexual organs on the gametophores can be induced by low-temperature treatment, and fertilization is facilitated by irrigation of the plant with distilled water. Meioses in the diploid sporophytes, which develop from fertilized eggs, give rise to haploid spores.

Although we are studying the development of *P. patens*, mutations leading to auxotrophy or resistance to toxic substances are necessary to identify linkage groups and to use as forcing markers in crossing or parasexual hybridisation experiments. Many auxotrophic mutants have now been isolated by a total isolation procedure (3,5). Morphologically abnormal mutants occur at a relatively high frequency after mutagenesis of spores (5,6) and can provide a source of material for study.

Exogenously supplied auxins and cytokinins affect many aspects of gametophytic development in mosses (7) and at high concentrations they inhibit growth. This effect has been used to find mutants of *P. patens* with decreased sensitivities to these substances (6); most of these mutants are also morphologically abnormal. Some non-selectively isolated mutants also show altered sensitivities to auxin and cytokinin. Mutants with altered sensitivities fall into a number of distinct categories, although there is some heterogeneity in morphology and sensitivity to auxin or cytokinin within these categories. Category 1 mutants grow to produce nearly spherical plants which consist almost entirely of chloronemata and are resistant to cytokinin and auxin. Category 2 mutants have about the same number of caulonemata as control strains, but they overproduce chloronemata and fail to produce gametophores. They are resistant to NAA (1-naphtylene acetic acid) and repaired (become more like wild-type i.e. in this case bud production is restored to control levels and chloronemata production is reduced) by BAP (6-benzyl aminopurine). Category 3 mutants have a similar morphology to the wild-type on minimal medium, but they are much less sensitive to NAA than control strains, although they respond similarly to wild-type to BAP. Category 4 mutants grow to produce a flat plant which consists mostly of chloronemata ,they produce no gametophores and only a few abnormal caulonemata. They are resistant to BAP, and are repaired by NAA. Category 5, 6 and 7 mutants are similarly resistant zo BAP and repaired to some extent by NAA, but they differ in the amounts of chloronemata, caulonemata and gametophores that they produce. The most likely explanation of mutant categories 4, 5, 6 and 7 is that they are defective to different extents in the synthesis of an endogenous auxin, category 4 mutants making the least amount of auxin. As a consequence of their inability to synthesise sufficient auxin, these mutants are resistant to BAP, indicating that auxin is necessary to allow the effects of endogenous or exogenous cytokinin. Conversely, category 2 mutants might be affected in the synthesis of an endogenous cytokinin, cytokinin being necessary to allow the effects of endogenous or exogenous auxin. Category 1 mutants, which make only chloronemata, may be impaired in their response to auxin (which is required for normal caulonemata production in category 4 mutants), or they are blocked in the response to or synthesis of another as yet unidentified factor. Evidence in favour of the latter argument is that category 4 mutants (putative auxin non-synthesisers) and category 1 mutants are not morphologically identical, and wild-type grown under a constant flow of fresh medium resembles category 1 mutants (6) suggesting that if this factor exists it is readily leached out of the protonemata. Category 3 mutants develop similarly to wild-type, indicating that they can synthesise auxin and cytokinin, but that they may be unable to take up exogenous auxin. This further suggests that free diffusion of auxin through the growth medium may not be necessary for normal development to occur.

Thus both endogenous auxin and cytokinin appear to play important and interdependent roles in several steps of gametophytic development. Recent physiological studies indicate that this may also be the case in the moss *Funaria hygrometrica* (8).

GENETIC ANALYSIS BY SOMATIC HYBRIDISATION IN PHYSCOMITRELLA

At least two kinds of mutants have been isolated which produce more than the wild-type number of gametophores (9). Whereas, the wild-type produces about 500 gametophores per plant after five weeks of growth under routine culture conditionts, category 9 and 10 mutants produce two to fifty times this number, and the "gametophores" of category 9 mutants have an abnormal morphology. Category 9 mutants have been shown to produce many times more cytokinin than the wild-type and this can be extracted from the medium on which they are grown (10, 11). N^6-(Δ^2-isopentenyl) aminopurine (2iP) has been identified as the main cytokinin in the culture medium of representatives from each of the 3 complementation groups present in this category (see Results and Discussion); zeatin is also present. 2iP has also been detected in the culture medium of the wild-type at a level estimated to be 50-100 times less than that present in the mutants (11). It is not surprising, therefore, that their morphology is identical to that of wild-type supplied with relatively high concentrations of exogenous cytokinin. Also, if wild-type is cultured in the same petri-dish as a category 9 mutant, cytokinin overproduced by the mutant diffuses through the medium and increases the number of gametophores on the wild-type.

Mutant strains with altered phototropic responses, and others with altered geotropic responses, have been found (4). In order to find ageotropic strains, it was necessary to screen plants after a dark growth regime, since caulonema and gametophores grow negatively geotropically only in darkness. Aphototropic strains have lost the ability to respond to the direction of light, although other light stimulated responses of their tissues appear normal.

Almost all morphologically abnormal mutants of *P. patens* are sexually sterile. Cultures must therefore be maintained by vegetative propagation and genetic analysis by crossing is not possible. There are two ways of approaching this problem. Conditional mutants, such as temperature-sensitive mutants could be sought, or a system of genetic analysis which is not dependent on the sexual cycle could be developed. We were attracted to complementation analysis by protoplast fusion both because protoplasts from mosses had been shown to be capable of regeneration (12) and because at that time protoplast fusion had already been used successfully by other authors to regenerate hybrid plants (13, 14). By adapting available methods (16, 17) genetic analysis by protoplast fusion was found to be possible in our system.

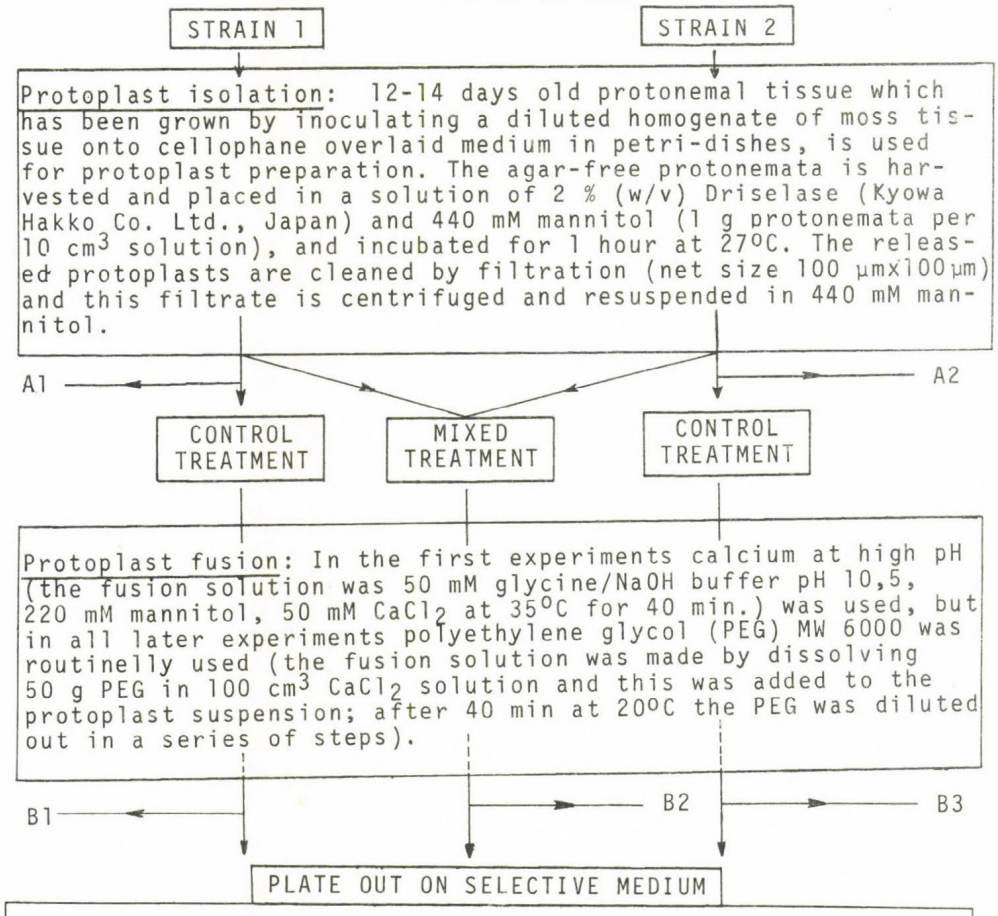

Figure 1 Design of protoplast fusion experiments

Control and mixed treatments are carried out in parallel. By taking small samples of the protoplast suspension where shown (A1, A2, B1, B2, B3) and plating these on appropriately supplemented media, the number of viable protoplasts entering the treatment and the number surviving the treatment can be estimated.

MATERIALS AND METHODS

Nearly all of the materials and methods used in these studies, including culture conditions and the isolation and characterisation of the strains used have already been published in detail (3, 4, 6, 9, 18, 19). However, an outline of some of the methods we use in protoplast work is given in Figure 1 for reference, and an explanation of the genetic nomenclature used is included below.

Genetic Nomenclature

It is not possible to establish directly by crossing, that the phenotype of a developmentally-abnormal strain which is sexually sterile, results from a mutation in a single gene, although this can be established in some cases, following genetic analysis using hybrids produced by protoplast fusion. Throughout this paper, mutant alleles whose Mendelian behaviour has been established, are represented by lower case symbols, e.g. ove B100. Upper case symbols, e.g. OVE A201, are used to represent putative mutant alleles which lead to a stable phenotype. However, it must be emphasised in these cases that, whilst it is simplest to assume that a developmentally-abnormal phenotype is caused by the possession of a single mutant allele, no critical demonstration of this use has been made.

For simplicity, throughout this paper, strains are referred to except where indicated otherwise, by their relevant partial genotype.

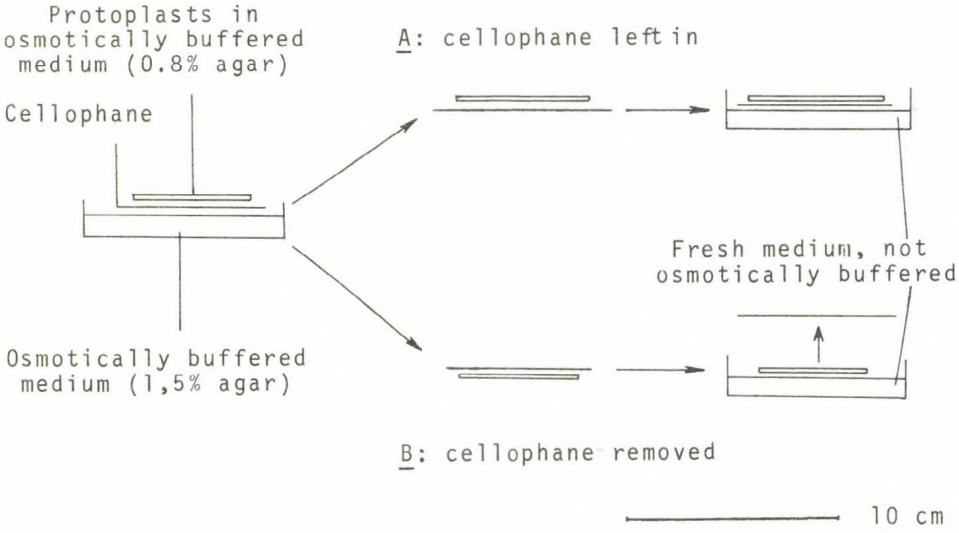

Figure 2 Cellophane transfer techniques

After a cell wall has developed, the regenerating protoplasts can be transferred to lower osmolarity medium on which they grow more quickly. In practice there is no difference between the two methods shown, as the cellophane is permeable to small molecules. Following transfer, the osmolarity on the top layer rapidly falls to that of the bottom layer. This method can also be used to transfer protoplasts between different chemical treatments, if desired.

RESULTS AND DISCUSSION

Much of the preliminary work on protoplast fusion has already been published (18, 19) and this is only discussed briefly in the following section; more emphasis is placed on work which has not been published.

Although calcium at high pH (16) was used successfully at first to induce protoplast fusion in *P. patens* (18) polyethylene glycol (19) was later found to be a more reliable technique, and was used for all the experiments described here. Before somatic hybridisation was applied to the study of developmentally abnormal strains, complementation analyses of various auxotrophic mutants were carried out in order to assess the technique. Six independently derived nicotinic acid auxotrophs were hybridized in all possible pairwise combinations and the results of this series of experiments is summarized in Table 1. In this analysis, the presence or absence of hybrids selected on medium without nicotinic acid was used to tell if complementation was occurring.

TABLE 1 Complementation analysis of nicotinic acid auxotrophs (Ref. No. 19)

Partial genotype	nic-2	nic-4	nic-5	NIC-6	nic-7
nic-4	0(-)				
nic-5	46(+)	75(+)			
NIC-6	55(+)	42(+)	0(-)		
nic-7	22(+)	21(+)	191(+)	560(+)	
nic-10	0(-)	0(-)	600(+)	82(+)	182(+)

Each figure in the Table represents the number of hybrids obtained from separate experiments.

(+)indicates complementant.

(-)indicates lack of complementant.

Where no hybrids were found the experiment was repeated to make sure that this result was not obtained by chance. The absence of hybrids indicates that the strains involved carry mutations

in the same gene. These results can be summarized into complementation groups: -

- group A: nicA2 nicA4 nicA10
- group B: nicB5 NICB6
- group C: nicC7

A nicotinic acid auxotroph isolated more recently, NIC-8 (20) was later allocated to group B using somatic hybridisation (5).

Data obtained from crossing experiments (3, 21), where available, was in agreement with the above complementation analysis.

A similar analysis of p-aminobenzoic acid (pab) auxotrophs was then carried out (5). In this case outcrossed strains, which carried auxotrophic markers other than pab, were available, and it was possible to select for hybrids on medium which contained p-aminobenzoic acid, using these other markers to force hybridisation. Thus hybrids were always produced, and complementation could be tested by subculturing the hybrids onto medium with and without p-aminobenzoic acid to test their auxotrophy (see Table 2)

The analysis shown in Table 2 can be summarized into complementation groups:-

- group A: pabA3
- group B: pabB4 pabB5 pabB6

TABLE 2 Complementation analysis of p-aminobenzoic acid auxotrophs

Full genotypes of the strains used		No.of hybrids growing	Result of subsequent growth test for p-aba	Complementation for pab genes
1	2			
pab-3 nic A10	pab-4 nic B5	60	prototrophic	yes
pab-3 nic B5	pab-5 nic A10	10	prototrophic	yes
pab-3 nic B5	pab-5 nic A10	54	prototrophic	yes
pab-3 nic A10	pab-6 thi-1	3	prototrophic	yes
pab-4 nic B5	pab-5 nic A2	6	auxotrophic	no
pab-4 nic B5	pab-6 thi-1	66	auxotrophic	no
pab-5 nic A10	pab-6 thi-1	19	auxotrophic	no
pab-5 nic A2	pab-6 thi-1	3	auxotrophic	no

The assessment of the technique of somatic hybridisation using auxotrophic mutants allowed the morphology of a large number of hybrids to be examined. Three different classes of hybrid morphology were found to occur (see Table 3).

TABLE 3 The morphology of somatic hybrids produced by protoplast fusion between morphologically normal haploid strains of *Physcomitrella patens*

Type of hybrid	Frequency	Morphology
Class I	<1 %	almost identical to wild-type
Class II	50-80 %	1/4 to 1/2 of the wild-type number of gematophores; chloronemata and caulonemata contain more red/ /brown pigment
Class III	20-50 %	identical to class II hybrids, except no or very few (1/50 of the wild-type number) gametophores produced

Class II hybrids have been self-fertilized (5, 18, 21, 22) and class I hybrids have also been selfed (5); all the data resulting from the segregation of markers amongst the progeny are consistent with their being diploids. The F1 plants so obtained again show these different morphologies, but with different frequencies between the progeny of different sporophytes, and selfing F1 plants gives segregations consistent with the F1 being diploids (5). We do not know why the morphological distinction between class I and class II hybrids exists, and this problem awaits detailed cytological investigation. Class III hybrids, which make no or very few gametophores and are therefore very difficult to self, may be of a higher ploidy level (>3n). Evidence in favour of this is that when class II hybrid morphology plants are hybridised to haploid plants all of the resulting hybrids have class III morphology (5, 19).

The finding that 3 different classes of hybrid occur must be taken into consideration when a complementation analysis of morphologically abnormal strains is done. If the presence or absence of gametophores is being investigated in the hybrids, enough plants must be examined to be sure that they are not all class III.

A clear-cut step in the development of *P. patens* is the formation of buds on the caulonemata. An attempt was made to isolate non-selectively mutants which had a normal protonemal morphology but which failed to produce buds. Since many studies (7) indi-

cated the involvement of cytokinin in this step in mosses, we believed that these mutants might be unable to produce cytokinin. However, although about 40,000 clones of mutagenized spores were screened, no plants with exactly this morphology could be found. The reason for this failure became clear when studies with auxin and cytokinin resistant mutants began, and it was found that cytokinin was also important for other earlier stages of development. Although no cytokinin non-producing strains were isolated in this particular study, many mutants which were blocked very early in gametophore development (GAM strains) were found. These mutants produced more secondary chloronemata than wild-type, and the buds grew to make only a cluster of cells; no shoots or leaves were produced. More careful study using different media and growth regimes revealed that there was heterogeneity in this class of mutants; GAM-355 for example, did not grow in the dark, and there were differences between mutants in the number of cells produced in the buds. A complementation analysis of these strains was attempted (5). Selection of hybrids was done using the auxotrophic markers thi-1 and pab A3, and subsequently a number of hybrids from each complementation test were examined for gametophore production (see Table 4).

TABLE 4 Complementation analysis of GAM strains

GAM-strains isolated from pab A3	GAM-strains isolated from thi-1					
	thi-1, control	26	7/10	7/13	355	363
pab A3 control	control	r	nt	r	C	nt
121	nt	+*	+*	+	nt	+*
139	r	+	+*	nt	+	+
173	r	+	+*	-	nt	+*
196	nt	nt	+*	+*	+	+*
224	r	+	+*	+	nt	+*
280	r	+	+*	+	nt	+*
295	r	+	+*	-	nt	+*

\+ indicates that the strains complemented

\- indicates that the strains did not complement

nt indicates that the complementation test was not done

C indicates codominance (Strain 355 carries a codominant mutation)

r indicates that the mutant strain was found to be recessive

* These hybridisations are also dominance tests, showing that GAM-7/10, GAM-363, GAM-121 and GAM-196 carry recessive mutations.

Since GAM-7/13 did not complement with either GAM-173 or GAM-295, it is predicted that GAM-173 would not complement with GAM-295, but a complementation test between GAM-295 and GAM-173 cannot be done (since they were both isolated from pab A3 spores). This serves to illustrate that although the number of complementation test which can be done using this system is limited, as long as enough morphological mutants of a certain type can be isolated from each auxotrophic strains, the number of genes in this step may be found.

Strains which were abnormal later in gametophore development (GAD strains) were also found in the non-selective screening mentioned previously. One type of these produces small leaves in which the pattern of cells is irregular, and the results of an analysis of these is shown in Table 5.

TABLE 5 Complementation analysis of GAD strains (21)

GAD strains isolated from pab A3	GAD strains isolated from thi-1			
	thi-1 control	91	74	33
pab A3 control	control	r	C	r
284	C	+	-	+
66	r	-	+	-
186	r	-	+	-
48	D	nt	nt	D

D - indicates dominance. The other symbols are explained in Table 4.

Although GAD-48 is dominant and therefore cannot be analysed in this way, and GAD-284 and GAD-74 are codominant so that the hybrids formed using these strains as components were sometimes not straightforward to classify morphologically, the analysis can tentatively be summarised:

Complementation group A: GAD A66, GAD A186, GAD A91, GAD A33

Complementation group B: GAD B74, GAD B284

Strains have been isolated which, when grown in the dark, produce protonema which fails to show the negative geotropism typical of dark-grown wild-type protonema (4). Two of these (GTR-2 and GTR-3), when grown in the light, produced on their gametophores, leaves smaller than those of the wild-type, and rounder in overall shape. GAD strains had also been isolated which produced leaves of the same 'rounded' appearance. Parasexual hybridisation has been used to show that the mutations in GAD-106 (a typical rounded-leaf GAD) and in GTR-2 and GTR-3 are recessive to their wild-type alleles, and fail to complement with

each other (21). Another recessive mutation causing only ageotropism of dark-grown protonema complements with all three: -

Group A: GAD A106, GTR A2, GTR A3.

Group B: GTR B1.

The direction of incident light determines the direction of growth of many of the tissues of *P. patens* and mutants have been obtained which are abnormal in this response (4). Some mutants (ptr-1, ptr-2 and ptr-3) clearly show a loss of all phototropic responses in bright white light, and others (PTR-4, PTR-5) appear to have lost only protonemal phototropism. Although it has been established by somatic hybridisation that all of these strains carry recessive mutations, detailed analysis has so far been restricted to ptr-1, ptr-2 and ptr-3 (21). Two complementation groups were found after somatic hybridisation experiments: -

Group A: ptr A1

Group B: ptr B2, ptr B3.

These strains could also be crossed, and the results were consistent with the complementation analysis, ptr A1 recombined freely with either ptr B2 or ptr B3, whereas when ptr B2 and ptr B3 were crossed no recombinants were detected in about 2300 progeny.

Mutants which produce more than the wild-type number of buds (OVE mutants) have also been studied using protoplast fusion (22). Among 17 independently isolated category 9 (cytokinin overproducing) strains 13 were found to be recessive and one was dominant. Although the dominance tests and the complementation analysis are not yet complete, it has been possible to recognize at least 3 complementation groups: -

Group A: OVE A78, OVE A102, OVE A201, OVE A131

Group B: ove B100, OVE B300, OVE B302

Group C: ove C200

Dominance tests indicated that four category 10 OVEs were all recessive, and self-fertilization of the hybrids formed gave the expected segregation of the OVE phenotype, showing that the strains carried single mutations. A complementation analysis further indicated that the four strains all carried mutations in the same gene, thus the strains can be designated: -

- ove A90, ove A97, ove A99, ove A107A.

Whereas the wild-type produces gametophores singly, one stem arising from a point on a caulonemal filament, two mutants (OVE-59 and OVE-65) have been found which produce bunches of several gametophores instead of single gametophores, giving a 'multiple axis' or 'leafy' appearance to the plant. After prolonged periods of culture, these 'leafy' strains occasionally gave rise to sectors which contained more red-brown pigment in the protonema and produced sporophytes apogamously in place of gametophores. Spores produced by these sporophytes could ger-

minate and give rise again to the 'leafy' variety. Since appropriate forcing markers were available, it was possible to do a complementation analysis. OVE-59 was found to be recessive and OVE-65 was found to be dominant. Most of the OVE-65/OVE-59 hybrids were apogamous sporophyte producers. These results suggest that the apogamous sporophyte producing strains are diploid varieties of the 'leafy' strains; further evidence for this hypothesis is that regular tetrads have been observed in the OVE-59/ /OVE-65 sporophytes (23).

The analysis of auxin and cytokinin resistant mutants (categories 1-8) using protoplast fusion has only just begun. It will be possible to obtain many more of these mutants using selective isolation techniques. Some of the results presented below (see Table 6) have already been published (6). Although a relatively high proportion of the mutants contain dominant mutations, many contain recessive mutations and can be analysed using somatic hybridisation.

TABLE 6 Results of dominance tests on auxin and cytokinin resistant strains using protoplast fusion

Category	Possible physiological defect	Strains	Dominant/Recessive
1	Not known (see ref.7)	NAR-91, NAR-112 NAR-113	dominant codominant
2	Cytokinin synthesis	NAR-87, GAM-429	codominant
3	Auxin uptake	NAR-171, NAR-180	recessive
4	Auxin synthesis	BAR-1, BAR-2, BAR-297	recessive
5	Auxin synthesis	BAR-130	recessive
6	Auxin synthesis	BAR-61 BAR-330	recessive codominant

SUMMARY

Biochemical and developmentally abnormal mutants of the moss *Physcomitrella patens* can be obtained following mutagenic treatment of spores. Many biochemical mutants can be analysed genetically by crossing, but most developmentally-abnormal mutants are sexually sterile and an alternative method of analysis has therefore been devised using fusion of isolated protoplasts to obtain parasexual hybrids. Segregation of markers in progeny from self-fertilization of those hybrids is consistent with them being diploid. To show that hybrids of this kind could be used for genetic analysis, complementation analyses of seven nicotinic acid auxotrophs and of four p-aminobenzoic acid auxotrophs, all of independent origin, were carried out; crossing data, where available, confirmed these results. The hybrids

formed following protoplast fusion of morphologically normal strains fall into three morphologically distinct classes.

Complementation analyses of developmentally-abnormal mutants can be done using auxotrophic mutations carried by the strains to select for hybrids. Many OVE mutants produce much more cytokinin than the wild-type and hybridisation experiments indicate that at least three genes can mutate to give this phenotype. At least two genes can mutate to give an aphototropic phenotype; in this case both parasexual hybridisation and conventional genetic techniques can be used to analyse these recessive mutations. Strains have been found which have an altered response to gravity, and these have been analysed using protoplast fusion. Apogamous sporophyte producing strains result following the hybridisation of a certain type of mutant which is abnormal in gametophore production. Hybridisation experiments have also shown that some auxin and cytokinin resistant mutants contain dominant mutations, while others are recessive and are amenable to complementation analysis.

We believe that *Physcomitrella patens* offers an excellent opportunity to study plant development. The plant hormones, light responses, photosynthesis and developmental problems can be studied in a system which allows the application of biochemical techniques. Somatic hybridisation following protoplast fusion plays a key role in our studies; without it genetic analysis of many developmentally abnormal mutants would not be possible.

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ISOLATION OF SUBPROTOPLASTS FOR GENETIC MANIPULATION STUDIES

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ABSTRACT

Subprotoplasts (enucleated protoplasts = cytoplasts, nuclei-containing subprotoplasts = karyoplasts and miniprotoplasts) can be isolated by different methods, but most effectively from protoplasts of cultured cells by high speed centrifugation in density gradients. Solutions of salts, sugars and modified silica gels are used to establish isoosmolal gradients of different densities which are not toxic to protoplast cultures. Whereas cytoplast preparations are extremely pure, enucleated subprotoplast populations, the karyoplast preparations consist of evacuolated miniprotoplasts,still with a considerable amount of the original cytoplasm. When transferred to appropriate culture conditions, some of the karyoplasts develop into "normal" protoplasts and later form cell colonies. Organelle transfer studies via subprotoplast fusion are restricted, therefore, to clearly defined and effective selection systems.

INTRODUCTION

Protoplast fusion and somatic hybridisation have been used very effectively in the last few years for fundamental studies in plant somatic cell genetics as well as for applied agriculture-oriented studies (several reviews in 1). In addition to fusion of two types of whole protoplasts, somatic combination of separate parts of the plant cell genome is of special interest. Somatic combination of different types of extranuclear characters or the transfer of cytoplasmic characters to another nuclear genome background can be achieved also by protoplast fusion when proper markers and selection conditions are used (2,3). To improve this chance, formation of special nuclear and plastidal genome combinations, Zelcer et al. (4) prepared physiological subprotoplasts by X-ray treatment and thereafter transferred cytoplasmic male sterility by protoplast fusion. Separate transfer of the nuclear genome, plastome and chondriome was originally approached by the transfer of isolated cell organelles. Although various methods for the transfer of isolated nuclei and chloroplasts into higher plant protoplasts have been

described, final proof for integration, expression and multiplication of transferred organelles is still lacking (5,6). Possible reasons for the failure of this type of experiment with plants may be the toxicity of transfer conditions for isolated organelles or uptake by phagocytosis which prevents rapid integration into receptor cells. The use of membrane-coated organelles is an alternative approach, e.g. by coating isolated organelles in synthetic membranes such as phospholipids (7). Another possibility is to preserve the original protoplast membrane and to prepare subprotoplasts by protoplast fractionation. Thus nuclei containing subprotoplasts (karyoplasts or miniprotoplasts) and enucleated subprotoplasts (cytoplasts) can be prepared.

Subprotoplast Isolation

Naturally formed subprotoplasts

Subprotoplasts are formed naturally by different processes, e.g. in the pericarp of some Solanaceous species, during plasmolysis of elongated cells, and by budding of protoplasts in culture. Subprotoplasts isolated from ripening fruits of Solanaceae have been described and used in fusion experiments by Binding (8).

Formation of enucleated and nuclei-containing subprotoplasts caused by plasmolysis of elongated cells is a very common phenomenon depending upon physical parameters of the cell structure. We used pollen of Hyoscyamus muticus and Nicotiana tabacum to prepare subprotoplasts enzymatically from germinating pollen tubes. Pollen grains were incubated in pollen medium (0.44M sucrose, 1.3 mM $CaNO_3$, 1.6 mM boric acid, pH 6.5) to induce tube growth and an equal volume of enzyme mixture (1% cellulysin, 1% pectinol, 1% driselase, 0.2 M mannitol, 5 mM $CaCl_2$, pH 5.7) was added after 30 min. By this treatment, a high number of protoplasts and subprotoplasts of different sizes were isolated. Of special interest with this type of material is the haploidy and the chance that subprotoplasts containing only the generative nucleus are naturally-occurring karyoplasts. Pollen protoplasts are being used now in culture and fusion experiments (Lörz, unpublished).

Subprotoplast formation by budding is a frequently observed but normally undesired phenomenon in protoplast cultures. This event can be induced in Zea mays internode protoplasts in a high percentage when cultures are kept in a medium with high osmotic value (above 1000 mOs/kg H_2O) for about two to three days. This method, however, was not effective in other cultures. All the procedures mentioned above are effective only with specific plant material and of reduced applicability in general for subprotoplast isolation.

Subprotoplast isolation by centrifugation

Two populations of subprotoplasts can be routinely isolated by centrifugation of protoplasts. High speed centrifugation of mesophyll protoplasts in step density gradients produces evacuolated and enucleated subprotoplasts (9,10). The result is a

separation of the large vacuole and some cytoplasmic material from a heavier fraction containing the nucleus, most of the chloroplasts and bulk of the cytoplasm. Subprotoplasts of this type are, however, of low value in envisaged genetic manipulation studies.

More promising material for the isolation of cytoplasts with the bulk of the cytoplasm, and karyoplasts (nuclei surrounded by a small amount of cytoplasm only) are the highly cytoplasmic, non-vacuolated or at least slightly vacuolated protoplasts from meristems or cultured cells. For the preparation of subprotoplasts step density gradients can be established by very different mixtures of salts, sugars and modified colloidal silica (11,12). Of special suitability are solutions which are not toxic to protoplasts, which are impermeable to protoplast membranes, and mixtures which allow the establishment of isoosmolal gradients over a wide density range. All these prerequisites are fulfilled by using mixtures of Percoll (Pharmacia, Sweden) and mannitol (Table 1). Freshly isolated protoplasts

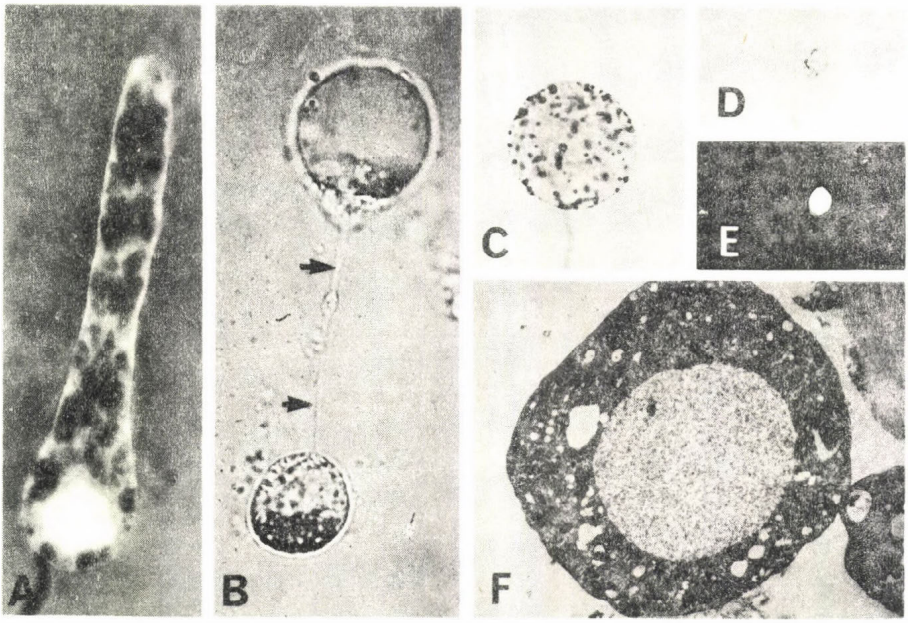

Fig. 1. Isolation of subprotoplasts by centrifugation.

A) Hordeum vulgare mesophyll protoplast with fluorescent stained nucleus after 20 min centrifugation at 20,000 rpm.
B) Separation of subprotoplasts from a Zea mays internode cell. A thin cytoplasmic bridge (arrows) is still present.
C,D,E) Protoplast and karyoplast from cultured cells of Nicotiana tabacum (E = D in fluorescent light).
F) Electron micrograph of a nucleus containing subprotoplast from a cultured cell of Zea mays.

TABLE 1 Preparation of subprotoplasts from corn cell-line protoplasts in an isoosmolal step density gradient

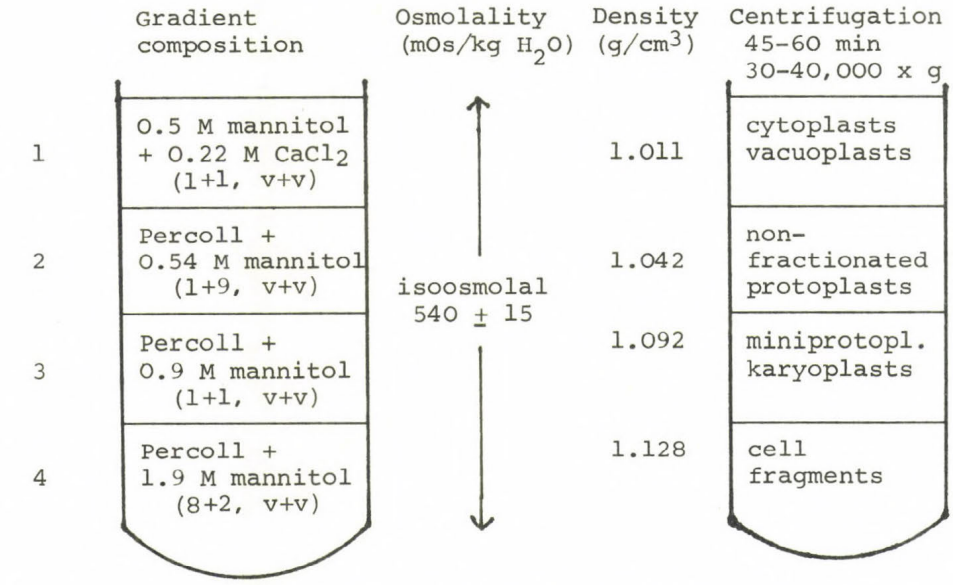

TABLE 2 Size and development of subprotoplasts isolated by centrifugation from corn cell-line protoplasts

	PROTOPLASTS (control)	CYTOPLASTS	KARYOPLASTS
after centrifugation			
DIAMETER (µm)	49.4 ± 4.2	47.2 ± 10.9	24.8 ± 1.3
VOLUME (%)	100 ± 28	87.2 ± 37.5	12.6 ± 2.1
1 day in culture			
DIAMETER (µm)	52.5 ± 5.4	49.4 ± 6.3	36.1 ± 2.0
VOLUME (%)	120 ± 41	100 ± 43	39.0 ± 6.9
2 days in culture	first cell divisions	20-50% viable some budding	40-80% viable cell enlargement
4 days in culture	20-30% dividing cells	< 5% viable	singular cell divisions

are put on top of the gradient and fractionated by centrifugation at 20,000 rpm for 1 h. Cytoplasts and vacuoplasts float on top of the gradient. Some non-fractionated and small protoplasts are found distributed in gradient steps 2 and 3, while karyoplasts and miniprotoplasts band between step 3 and 4. Cell debris and fragments pellet to the bottom of the tube. After collection from the gradient the subprotoplast fractions are washed with osmoticum or medium to remove Percoll. Use of cytochalasin B in our experiments did not show a significant increase in subprotoplast yield.
The size of subprotoplasts isolated from corn cell-line protoplasts (13) has been determined after isolation and after transfer to culture medium (Table 2). Karyoplasts have about 10-15% of the volume of the original protoplasts. When kept in culture they increase rapidly in size and in a very few cases cell divisions are observed. The viability of cytoplasts in culture decreases and no survivors were observed after 7 days. The cytoplast fraction seems therefore to be a pure population of enucleated subprotoplasts and useful material to transfer nuclear-independent genetic information. The observation of a low number of developing cells in the karyoplast fraction is strong evidence that some of the subprotoplasts are miniprotoplasts still containing enough cytoplasmic material to allow normal development with about 2 days' retardation compared to control material. This is also in accordance with the results of Wallin et al. (14) who obtained hybrids when mini-protoplasts of complementing tobacco mutants were fused. On the other hand the use of karyoplasts as an alternative to nuclei transfer is restricted because additional selective systems are absolutely necessary to exclude "contamination" of subprotoplasts with non-fractionated protoplasts.

Fusion studies with subprotoplasts

Bearing in mind the restrictions mentioned above, only fusion experiments with protoplasts having well-defined markers and based on effective selection systems seem to be meaningful. We concentrate our efforts therefore on material which enables us to discriminate between fusion products formed by PROTOPLAST + CYTOPLAST (cybrids) or PROTOPLAST + KARYOPLAST (nuclear hybrids) or PROTOPLAST + PROTOPLAST (fusion hybrids).
This possibility is opened up by Nicotiana tabacum mutants bearing two mutations: a nuclear gene coded chlorophyll deficiency and as a non-mendelian character the streptomcycin resistance (15). Subprotoplasts of this material in combination with nitrate reductase deficient mutants (16) should allow us to find conclusive results on the usefulness of subprotoplasts in fusion experiments. In addition, subprotoplasts may be advantageous in studies of cellular incompatibility, induction of vigorous growth pattern of hybrids (17), fusion experiments with small objects such as yeast protoplasts, or in gene transfer studies by fusion of subprotoplasts isolated from micronucleated cells.

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THE PHYSIOLOGY OF FUSION IN ISOLATED HIGHER PLANT PROTOPLASTS AS A BASIS FOR SOMATIC CELL GENETICS

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In 1972, during the International Protoplast Symposium held in Versailles, professor Cocking said the following: "Workers are understandably preoccupied with the desire to produce the new somatic hybrid plant and are sometimes not prepared to study in detail the various stages necessarily required for this novel approach to plant breeding to be fully implemented. This is not to say that the 'hit or miss' approach will not be rewarding in giving some form of result but only a systematic study of each stage in this multi-stage process will enable a basic foundation to be laid" (1). In the present paper, following Cocking's demand, I want to draw your attention to some specific problems related to fusion events.

During the last years, remarkable success has been achieved in the fusion of higher plant protoplasts as well as in the recovery of plants from fusion products (2-5). In the early work, inorganic salts, especially sodium nitrate, have been used as fusion inducing agents (6). Later, Keller and Melchers (7) proposed the application of a high calcium/high pH medium to induce protoplast fusion. Kao (8) and, nearly at the same fime, Eriksson (9) introduced the use of polyethylene glycol (PEG) as a powerful fusogenic agent. Since then, PEG has been successfully used to fuse both plant and animal cells (10-13). The fusion of membranes is a prerequisite of the fusion of (animal) cells and plant protoplasts. In contrast to the membranes of animal cells (14), our knowledge of the plant cell membrane, the plasmalemma, is scanty. Recently (15), an interesting study was performed on the phospholipids of cell membranes of various plant species cultured in suspension, with regard to the fatty acid composition of the membranes. This composition was found to be largely independent of the plant species. At the present stage, we have to assume that the structure of plant cell membranes is similar to that of the animal cell membranes.

The cell membranes consist mainly of lipids and proteins. The phospholipids are negatively charged in the physiological pH range thus giving a negative charge to the whole cell membrane, which is a severe problem in any fusion event. Among the membrane proteins, the glycoproteins are of particular interest.

They carry oligosaccharides on the N-terminal end, directed towards the surroundings of the cell. These carbohydrates are of special interest as to the problem of recognition and adhesion of membranes. Furthermore, they are the binding sites of lectins and viruses.

Assuming we possess a mixed population of protoplasts A^- and B^-, both kinds of protoplasts are loaded with negative charges, resulting in mutual repulsion. If these charges are neutralized an adhesion of the protoplasts can be achieved (to reach this effect, the neutralization of the charges of one protoplast species would be sufficient).

For a long time, it was supposed that the treatment of protoplasts with calcium ions will compensate the negative charges of the plasmalemma. This assumption has been proved to be correct in 1978 by Nagata and Melchers (16). 100 mM Ca^{++} decreased to zero the electrophoretic mobility (U) of tobacco protoplasts and the corresponding ζ-potential* directly proportional to U. By this treatment the aggregation of protoplasts was greatly increased. The effect of calcium ions, however, is not restricted to the neutralization of electric charges. The Ca^{++}-ions penetrate much deeper into the cell membranes. From the results of investigations on phosphatidylserin-liposomes, Papahadjopoulos et al. (17) conclude that the fusion energy may come from lipid-protein phase separations induced by calcium ions. Liposomes do fuse preferentially near or somewhat above the liquid-crystalline transition temperature. In this way, so-called smooth membrane surfaces with increased lipid fluidity are formed. These participate in the first membrane contacts.

The low level hydrogen ion concentration may lead to the formation inside the membrane of lysophospholipids (e.g. lysolecithin) which are known to initiate fusion in animal cells.

The mechanism of the membrane fusion initiated by PEG is as yet unknown. There are a number of hypotheses that may be summarized as follows. PEG is a polycondensate of ethylene glycol. The macromolecule has a negative charge. Various low and high molecular weight hydrophilic substances can be bound to PEG by means of their positively charged groups. When the chain is long enough, it can serve as a bridge between the cell membranes of adjacent protoplasts. Of course, PEG can bind also cations, e.g. calcium ions. Thus, it is possible that bivalent cations constitute a link between the negatively charged groups of phospholipids and the negatively charged structures of PEG. During the

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* $\zeta = \frac{4\pi\eta U}{\varepsilon}$, where ζ = ζ-potential (mV),

U = electrophoretic mobility, η = viscosity,

ε = dielectric constant.

elution process of Kao's method (8), if high calcium/high pH solution is added (7), a possible redistribution of charges and formation of calcium bridges may lead to the fusion of membranes.

The elimination of the negative surface charges results in the formation of electrically neutral units which can be combined in the presence of aggregation promoters to pairs: AA, AB, and BB. A significant increase in the yield of heterokaryons may, however, be obtained by retaining the originally negative charges of one partner and changing the charges of the other. This treatment favours the combination $A^- B^+$. According to Nagata and Melchers (16) this can happen upon the addition of polycations. This results in the preferential combination of A^+ and B^-, or vice versa, in high frequencies. However, there are two difficulties. Firstly, all tested polycations, except protamine sulfate, have been found to be toxic to the protoplasts; secondly, the charge conversions are reversible after removal of the polycations from the medium. The permanent presence of the polycation, however, leads to the same charge conversion in the partner system and abolishes the desired effect. Therefore, a method must be looked for, resulting in a non-toxic, stable conversion of the surface charges of the cell membrane. Melchers reported results of experiments aimed at influencing the surface charges of protoplasts by positively charged synthetic phospholipids not only to eliminate these charges but to convert them into positive ones and to establish in this way a "secondary artificial sexuality" which may facilitate the fusion of membranes (18).

A sexual cell can recognize specifically the partner cell. This has not been reproduced so far by using the protoplast fusion technique. All fusion methods are quite non-specific; they represent physicochemical systems which also work with non-biological materials (e.g. incorporation of latex particles into protoplasts, etc.). In any case, the reactions do not seem to be physiological ones. They should be replaced as soon as possible by more biological methods.

In cell recognition the oligosaccharides of glycoproteins seem to be important. They are responsible for recognition and adhesion. In view of the highly specific structure of these sugars, experimental modification of the plasmalemma may be a method for aggregating cell membranes of only specified protoplasts. In this connection, the use of immunological methods (20), lectins, particularly Concanavalin A (Con A), seems to be extremely valuable (21-251). After Eriksson's (21) observation showing that Con A agglutinates the carrot protoplasts, and this can be inhibited by the addition of α-methylglucose to the medium, Con A has been used in the PEG fusion method for improving the fusion frequency (22).

Despite a series of electron microscopic investigations (23-25) with the aim of elucidating the linkage site(s) of Con A in the membrane, the molecular structures involved in this reaction have not yet been identified. It has been shown, however, that Con A is linked to the plasmalemma but not to the cell wall. Furthermore the linkage sites have been shown to be blocked

competitively by freshly formed cell wall precursors. Con A linkage sites may be blocked or destroyed also by toxic contaminations of the enzyme preparations used for protoplast isolation; a rapid restoration of these linkage sites, however, in a sorbitol containing solution could be observed.

I wish to draw your attention to an area, in which nature itself presents examples of directed cell fusion: the formation, agglutination and fusion of isogametes of the green algae, especially Chlamydomonas.

In conclusion, a number of problems have to be solved before a directed fusion of different protoplasts can be achieved.

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DNA TRANSFER EXPERIMENTS WITH PLANT PROTOPLASTS AND BACTERIAL PLASMIDS

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INTRODUCTION

The subject of DNA-mediated transformation of eukaryotic cells had undergone dramatic changes during the past two years. Although it had been suggested a long time ago (for review of these earlier results see 1) that non-viral exogenous DNA could be taken up by eukaryotic cells and expressed, it was only in 1978-79 that sophisticated genetic systems allowed a clear-cut demonstration of this phenomenon.

The results referred to above were obtained with yeast and several mammalian cultured cell lines (2, 6 and Table 1).

TABLE 1 Some eukaryotic systems in which transformation and/or transfection have been observed at the cellular level

Receipient cells	Induction of uptake	Donor DNA
Yeast	Protoplasts, $CaCl_2$, PEG	- Col E1-leuye recombinant plasmids (5) - Col E1-leuye-2u yeast plasmids (3)
*Xenopus laevis**	Microinjection	- SV 40 - Col E1-Drosophila histone genes (17)
Cultured mammalian cell lines	Calcium phosphate-DNA co-precipitate	- Viral DNAs (18) cDNA of oncornaviruses(19) - Herpes thymidine kinase gene (20) - SV40-hemoglobin cDNA SV40-λ hybrids (2,21) - Homologous and heterologous APRT genes(22)
Turnip protoplasts	?	- Cauliflower mosaic virus DNA (23)

*It is not known whether or not the added genetic traits are transmitted to the progeny.

These results could be obtained thanks to the convergence of apparently unrelated techniques such as the isolation and description of eukaryotic mutant cell lines and methods allowing efficient uptake of DNA by cells.

Such well-defined systems are not yet available in plants. Indeed, auxotrophic conditional lethal mutants in plants are restricted to the thiamine loci in *Arabidopsis* (7) and in the *Tomato* (8) or to the proline locus in *Maize* (9). Moreover, *Maize* protoplasts do not lend themselves to easy regeneration. On the other hand, suitable host-vector systems for introducing genetic information into plants cells still have to be produced (10).

Our aim is to develop techniques allowing to overcome the mechanical barrier to DNA uptake possibly presented by the cell wall and to investigate how the donor DNA can be protected against nuclease attack. For this, we used as recipient systems mesophyll protoplasts enzymatically isolated from *Vigna sinensis* (11) and *Nicotiana tabacum* SR1 (12,13). Plasmid DNAs were used as donors. Some of the advantages of these host-vector systems are listed in Table 2.

TABLE 2 Some properties of the recipient cells and of the donor DNAs used in our studies

Protoplasts	Plasmids
- High numbers of totipotent cells can be treated under standard conditions	- All molecules are identical
- Regenerating protoplasts can be cloned on selective medium	- Gene dosage per molecule is high
- Absence of cell wall theoretically facilitates penetration of macromolecules.	- Insensitive to exonuclease action
	- Cloning techniques allow amplification of selected genes.

One major disadvantage to the use of bacterial plasmids is that their functions are not expected to be readily expressed in a selectable way in plant cells (except, perhaps, for the Ti plasmid from *Agrobacterium tumefaciens*) (37). Nevertheless, they nicely fit the needs for biochemical studies dealing with the uptake of DNA and mimic cloning vectors to be used in future biological experiments with plants.

Several reviews devoted in part to DNA uptake studies in plant protoplasts and discussing the construction of potentially useful molecular vectors for plant cells have already been published (10, 14-16).

MATERIALS AND METHODS

Cowpea mesophyll protoplasts were obtained by enzymatic digestion of 7-day old primary leaves as described in (11). Tobacco shoots were grown aseptically on H medium without hormones (34).

Protoplasts were obtained as described in (13). (^{3}H)-p BR 313, (^{3}H)-p BR 322 and (^{3}H)-p CR1, plasmid DNAs were obtained after chloramphenicol amplification and centrifugation of cleared lysates in CsCl-ethidium bromide gradients (15).

In vitro recombination of pBR313 with barley DNA was performed with Bam HI digested DNA ligated with bacteriophage T4-induced ligase and introduced by transformation into *E. coli* C 600 (35). These experiments were carried out under P2 + EK1 containment according to NIH guidelines for recombinant DNA research.

Entrapment of plasmid DNA within liposomes will be described elsewhere (36).

Nucleic acids were purified from protoplasts by lysis with 2 % sodium dodecylsulfate, incubation with 100 ug/ul proteinase K for 1 hour at 37°C, phenol extraction and ethanol precipitation. Molecular sieving on Sepharose 4B and 6B was performed as before (31).

RESULTS AND DISCUSSION

1. Induction of DNA Uptake by Plant Protoplasts

Experimental conditions promoting the uptake and expression of other informative macromolecules - i.e. viral RNAs - have been worked out in detail (24, 25). Such conditions were also utilized in experiments where plasmid DNA was fed to higher plant protoplasts with the result that polycations and high pH values promoted irreversible binding of plasmids to protoplasts (26, 27). However, the plant species used in these studies yield protoplasts that do not easily regenerate mature plants. Therefore, additional experiments were carried out with tobacco SR-1 protoplasts displaying high totipotency.

Figure 1A represents the total binding of (^{3}H)-pCR1 (8.6 Mdal) DNA to tobacco protoplasts measured as the amount of radioactivity removed from the incubation medium as a function of time. It can be seen that incubation at pH 10.5 with $CaCl_2$ (25) generates the highest rate of binding after 30 minutes. Complex formation between pCR1 and poly-L-ornithine (26, 27) or histone HI from calf thymus in order to produce "minichromosomes" (28, 29) also increases the rate of binding compared to K_3 medium (13) alone. However, the analysis of the state of donor DNA in the medium by CsCl-ethidium bromide density gradients centrifugation indicates that plasmid complexed with histone HI or with poly-L-ornithine (not shown) is considerably degraded whereas incubation at high pH is much less harmful to plasmid DNA (Figure 1B). Nevertheless, only traces of supercoiled DNA are detected in the latter case, indicating that intense nicking activity occurs even at pH 10.5. Despite the fact that proto-

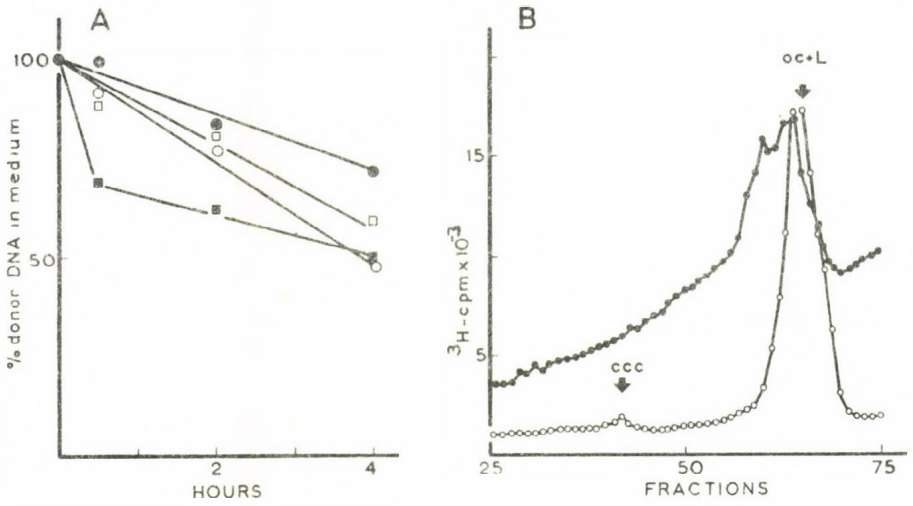

Fig. 1 A Withdrawal of (^{3}H)-pCR1 DNA from the incubation medium by tobacco protoplasts. ●—●, in K3M medium; □—□, histone HI-DNA complex in K3M medium; o—o, poly-L-ornithine-DNA complex in K3M medium; ■—■, in K3M medium at pH 10.5 in the presence of 50 mM $CaCl_2$.

B CsCl-ethidium bromide density gradient centrifugation of residual donor DNA in K3M medium (pCR1-histone HI complex), ●—● and in high pH medium, o—o after incubation with protoplasts for 4 hours and 30 min, respectively.

plasts incubated at pH 10.5 for 30 min. bind the same amount of (^{3}H)-pCR1 as protoplasts incubated for 3 hours in the presence of a (^{3}H)-pCR1 poly-L-ornithine complex, it is found that the amount of donor DNA irreversibly bound (DNase I - resistant) is 4fold higher at high pH (2.2 % of input versus 0.06 %). The physical state of these molecules was analysed by CsCl-ethidium bromide density gradient centrifugation.

Fig. 2A shows that donor (^{3}H)-pCR1 DNA consists of about 90 % supercoiled molecules. Plasmid DNA irreversibly bound to protoplasts after 30 min. at pH 10.5 was extracted according to the "cleared lysate" method as in (30) and banded in a CsCl-EB gradient.

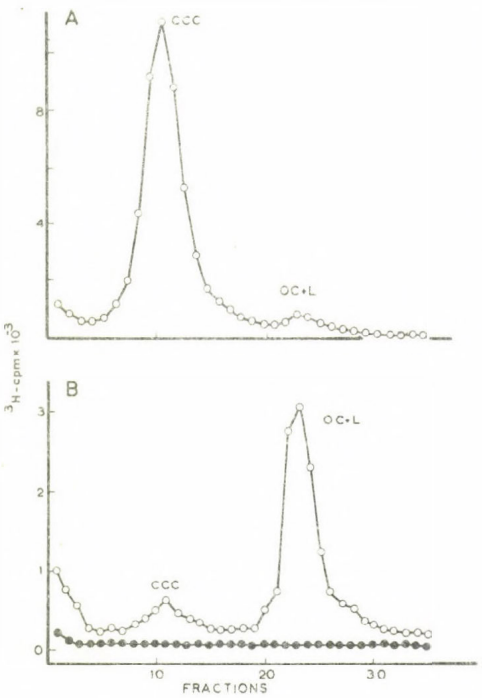

Fig. 2

A CsCl-ethidium bromide density gradient centrifugation of donor (^{3}H)-pCR1 DNA.

B CsCl-ethidium bromide density gradient centrifugation of DNA extracted from tobacco protoplasts incubated with (^{3}H)-pCR1 for 4 hours in K3M medium, ●—● and for 30 min. at pH 10.5 plus 50 mM $CaCl_2$, o—o .

Fig. 2B shows that supercoiled molecules were retrieved, most of the radioactivity banding at the position of nicked and linear DNA. When the incubation was carried out in unsupplemented K3M medium, a flat profile was obtained, indicating the absence of uptake of DNA able to band in a CsCl gradient.

The degree of polymerization of donor DNA irreversibly bound to tobacco protoplasts was further investigated by molecular sieving on Sepharose (31).

Figure 3 describes the molecular weight spectrum displayed by (^{3}H)-pBR 322 plasmid DNA (2.6 Mdal) found associated with protoplasts after a 45 min. incubation. As expected, incubation in K3M medium does not allow the recovering of intact plasmid molecules eluting at the void volume (fractions 9-11).

Heavy depolymerization of the donor DNA is also observed when polyethylene glycol and $CaCl_2$ are added to the medium in order to promote protoplast fusion (32) and compactization of DNA (33). The amount of retarded (degraded) DNA is decreased at the expense of excluded DNA when poly-L-ornithine and $ZnSO_4$ (26) or a combination of them with polyethylene glycol are present in the medium.

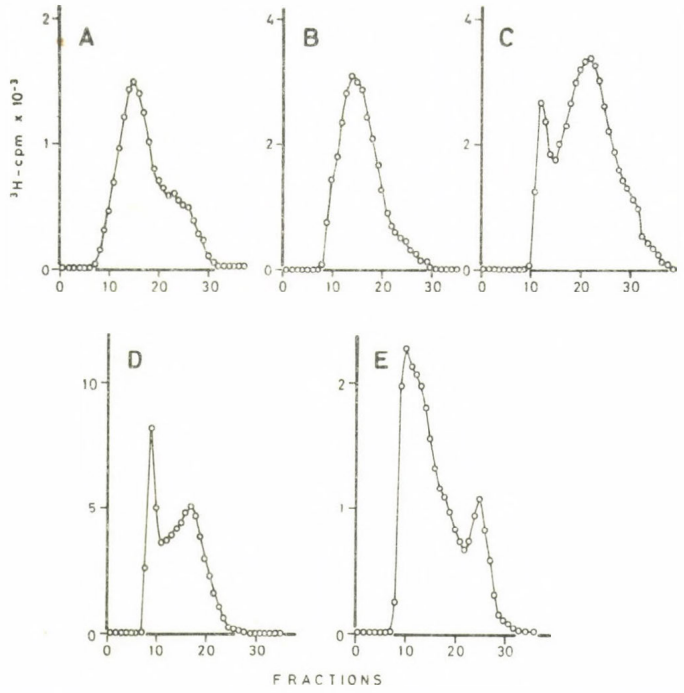

Fig. 3 Molecular sieving on Sepharose 4B of (^{3}H)-pBR 322 recovered from tobacco protoplasts after a 45 min. incubation in (A) K3M medium (B) in K3M medium plus PEG 6000, (C) in K3M medium with a poly-L-ornithine-pBR 322 complex in the presence of 5 mM $ZnSO_4$, (D) in K3M plus a combination of additives as in (B) and (C), (E) in K3M medium brought to pH 10.5 plus 50 mM $CaCl_2$.

Nevertheless, it appears that much more donor DNA is eluted at or close to the void volume when the incubation is carried out at pH 10.5 in the presence of $CaCl_2$. Thus, it seems clear that the latter conditions of incubation allow the best preservation of donor plasmid DNA in the medium as well as in the protoplasts. It should be noted that in all cases, protoplasts retained their ability to divide after the treatments. However, concentrations of DNA higher than 20 µg/ml become toxic at high pH.

2. Stability of Donor DNA After Uptake

It has been argued that covalently closed circular DNA molecules would constitute adequate molecular vectors for transferring new genetic information to plant cells (10, 14, 16, 38). In this regard, it was of interest to compare the fate of supercoiled and linear DNA fed to protoplasts. The plasmid pBR 322

possesses a single site of cleavage for the restriction endonuclease Bam HI (35).

The supercoiled plasmid can thus easily be converted into an identical but linear DNA molecule. Fig. 5 represents the analysis of supercoiled and linear pBR 322 irreversibly bound to cowpea protoplasts after a 45 min. incubation in the presence of $ZnSO_4$ and poly-L-ornithine (26).

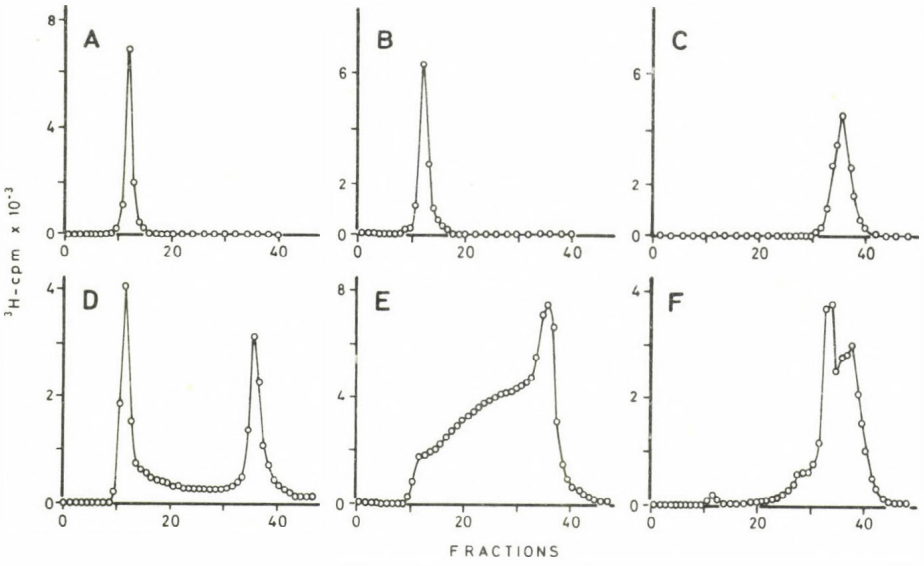

Fig. 4 Molecular sieving on Sepharose 4B of donor (^{3}H)-pBR and of (^{3}H) pBR 322 recovered from cowpea protoplasts after a 45-min. incubation. A Supercoiled pBR 322, B pBR 322 cleaved with Bam HI, C pBR 322 digested to completion by DNase I, D DNA recovered from cowpea protoplasts incubated with supercoiled pBR 322, E DNA recovered from cowpea protoplasts incubated with linear pBR 322, F Compounds recovered from cowpea protoplasts incubated with pBR 322 digested as in (C).

Figures 4 A, B and C represent the elution patterns on Sepharose 4B columns of supercoiled, linear (Bam HI-digested) and DNase I-digested pBR 322. As expected, supercoiled and linear plasmid molecules are excluded from the gel whereas the oligonucleotides produced by DNase I are strongly retarded. Figure 4 D clearly

demonstrates that supercoiled pBR 322 undergoes degradation in cowpea protoplasts but also shows the persistence of a considerable amount of polymerized plasmid molecules. On the other hand, linearized pBR 322 is degraded much faster than its supercoiled counterpart and displays a wide spectrum of molecular weights (Fig. 4) with a predominant peak eluting at the position of low molecular weight compounds (see Fig. 4C). This suggests that the free ends of linear DNA are highly susceptible to exonuclease activity present in the protoplasts.

Fig. 4 F shows that cowpea protoplasts are able to accumulate oligonucleotides produced by digestion of pBR 322 by DNase I prior to incubation. In this experiment, a total protoplasts lysate was applied on the column, therefore, the small amount of radioactivity eluted at the bed volume (fractions 11-13) represents aspecific trapping of low molecular weight products by cellular DNA. This was demonstrated by phenol extraction of these fractions and TCA precipitation. - No radioactivity was found, showing that there was no reutilization of DNase I-generated compounds for cellular DNA synthesis within the periods used.

In order to determine whether the radioactive compounds resulting from the degradation of supercoiled (^{3}H)-pBR 322 in cowpea protoplasts (right peak in Fig. 4D) still contain gene-size molecules (0.5 Mdal or more), the same extract was run on the less porous Sepharose 6B gel. On such a gel, DNA molecules of molecular weight 0.5 Mdal are also excluded (39, 40).

Fig. 5A shows that Sepharose 6B does not allow a clear-cut fractionation as in Fig. 4D. Instead, the excluded peak is followed by an important trailing indicative of a high degree of heterogeneity. Molecules eluted at the left of the arrow and representing ca. 45 % of the total radioactivity display molecular weight values higher than 0.4 Mdal (computed as in ref. 39) whereas those eluted at the right of the arrow (55 %) have molecular weights lower than 0.4 Mdal. These values compare well with those derived from Fig. 4D and thus show that about half of the plasmid recovered from the protoplasts is of sub-gene size.

Another important question is that of the stability of irreversibly bound DNA during periods of time allowing, e.g. cell wall regeneration and first cell divisions. We have previously shown (26) that plasmid DNA taken up by cowpea protoplasts does not undergo additional degradation during a 45-min. chase period. The same experiment was repeated with, however, a chase period extended to 48 hours after uptake - Fig. 5 B indicates that (^{3}H)--plasmid treated protoplasts release low M.W. DNA degradation products after transfer to fresh medium.

At the same time, the prominent excluded peak seen in Fig. 4D dramatically decreases at the expense of a large peak of intermediate M.W. DNA and a small peak of low M.W. compounds (Fig. 5C) perhaps corresponding to similar molecules excreted into the medium. Thus, the amount of high M.W. plasmid taken up drastically decreases with time. - Under these experimental conditions, cowpea protoplasts each take up approx. 14,000 copies of plasmid DNA (26). After 48 hours, only approx. 30 molecules per protoplasts are detected. These data also show that kinetic ex-

periments aiming at the determination of DNA uptake by protoplasts over extended periods (27, 37, 41) must deal with qualitatively different molecules as incubation times increase. Interestingly, it is also found that cowpea protoplasts fed with (^{3}H)-plasmid DNA degraded to completion by DNase I do not reutilize these products for their own DNA synthesis even after 48 hours (Fig. 5C).

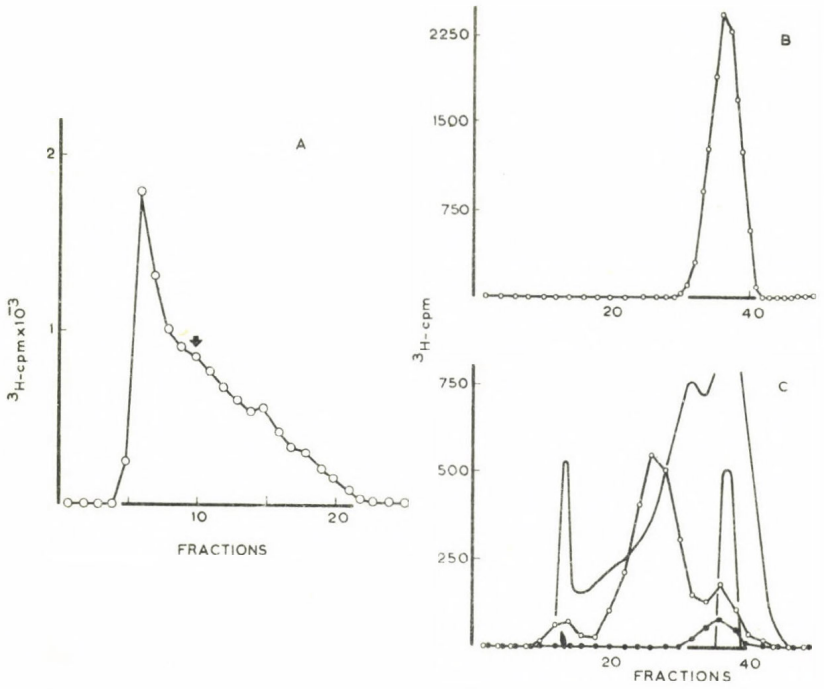

Fig. 5 A Molecular sieving on Sepharose 6B of DNA recovered from cowpea protoplasts incubated for 45 min. with supercoiled (^{3}H)-pBR 322-

B Molecular sieving on Sepharose 4B of compounds released after a 48-hour chase period by cowpea protoplasts incubated as in (A)-

C Molecular sieving on Sepharose 4B of DNA present in cowpea protoplasts after a 48-hour chase (treatment as in A), - , optical density at 254 min; o—o, (^{3}H)-radioactivity in protoplasts treated with supercoiled pBR 322; •—•, (^{3}H)-radioactivity in protoplasts treated with DNase I - digested pBR 322 (see Fig. 4C).

3. Donor DNA in Liposomes

Details concerning the entrapment of plasmid DNA of different MW in lecithin and lecithin/cholesterol liposomes as well as the interactions between the latter and plant protoplasts will be published elsewhere (36). Liposomes have previously been shown to be able to sequester DNA, to fuse with or be taken up by cultured mammalian cells, and finally to release their contents intracellularly so as to allow correct translation of the foreign message (42, 43). Thus, they seem to be promising carriers for the introduction of genetic information into plant protoplasts.

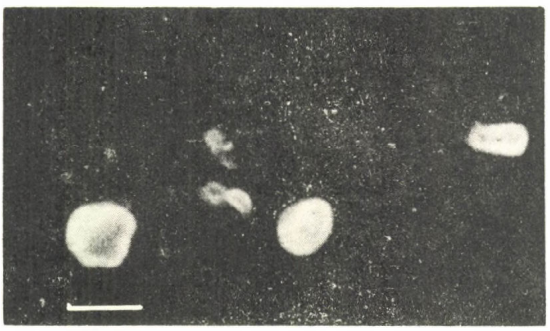

Fig. 6 Fluorescence micrograph of lecithin liposomes loaded with an *E. coli* DNA-ethidium bromide complex. Bar represents 2 µ.

Fig. 6 shows a fluorescence micrograph of liposomes containing a DNA-ethidium bromide complex. Such liposomes were loaded with (^{3}H)-pBR 322 and incubated with cowpea protoplasts either in Gresshoff-Doy medium alone or in the presence of polyethylene glycol 6000 in order to promote protoplast fusion (32). After 45 min. of incubation, the samples were gradually diluted with medium, the protoplasts spun down and extensively washed.

They were then lysed (26) and nucleic acids purified by incubation with proteinase K, phenol extraction and ethanol precipitation (36). Again, the molecular weight distribution of donor DNA associated with protoplasts was analysed by molecular sieving on Sepharose 4B.

Fig. 7A represents donor DNA associated with protoplasts after incubation of protoplasts with liposomes without addition other than culture medium. It can be seen that donor DNA is distributed between three fractions of high, intermediate and low M.W. If PEG 6000 is added to the incubation medium, the total amount of plasmid DNA bound to protoplasts is increased approx. 7fold, the proportion of high M.W. plasmid being much higher than in the absence of PEG 6000 (Fig. 7B).

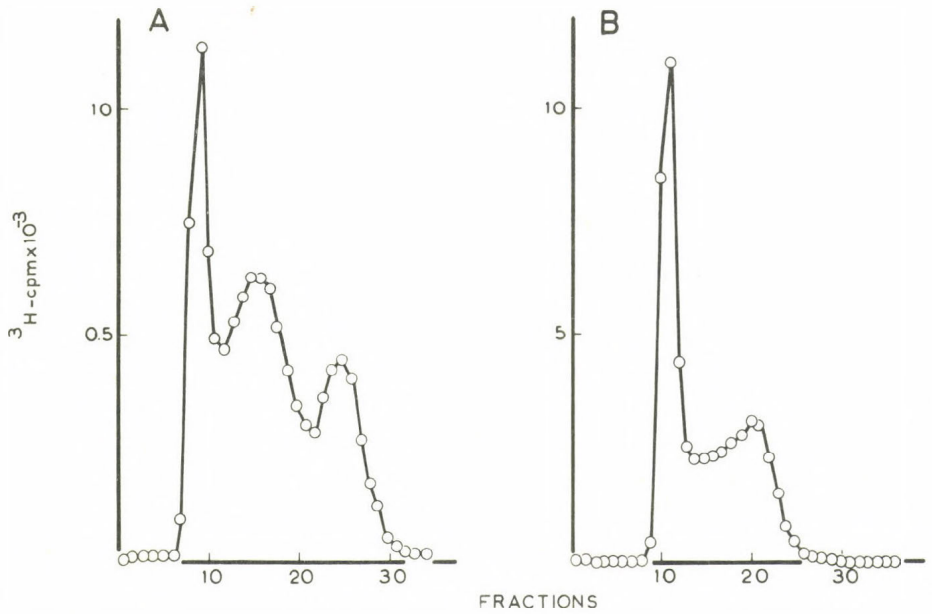

Fig. 7 Molecular sieving on Sepharose 4B of DNA recovered from cowpea protoplasts incubated for 45 min. with (^{3}H)-pBR 322 sequestered in liposomes. A Incubation in Gresshoff--Doy medium, B Incubation in medium plus PEG 6000.

Further experiments showed that part of this high MW DNA is associated with nuclei and consists of a majority of nicked plasmid molecules together with a significant proportion of supercoiled and linear molecules (36).

In order to assess the biological significance of this phenomenon, it might be of interest to determine whether TMV-RNA for instance could be expressed in tobacco protoplasts if sequestered in liposomes.

4. Construction of Vectors for Biochemical Studies

So far, uptake studies involving supercoiled DNA as donor were limited to bacterial plasmids. In order to compare the fate of defined stretches of plant and bacterial DNA in plant protoplasts, it was necessary to construct recombinant molecular vectors. This was done by "shotgun" digestion of barley DNA with Bam HI and *in vitro* recombination in the tetracycline gene of pBR 313. *E. coli* C 600 clones carrying recombinant plasmids were analysed for the size of the integrated barley DNA pieces.

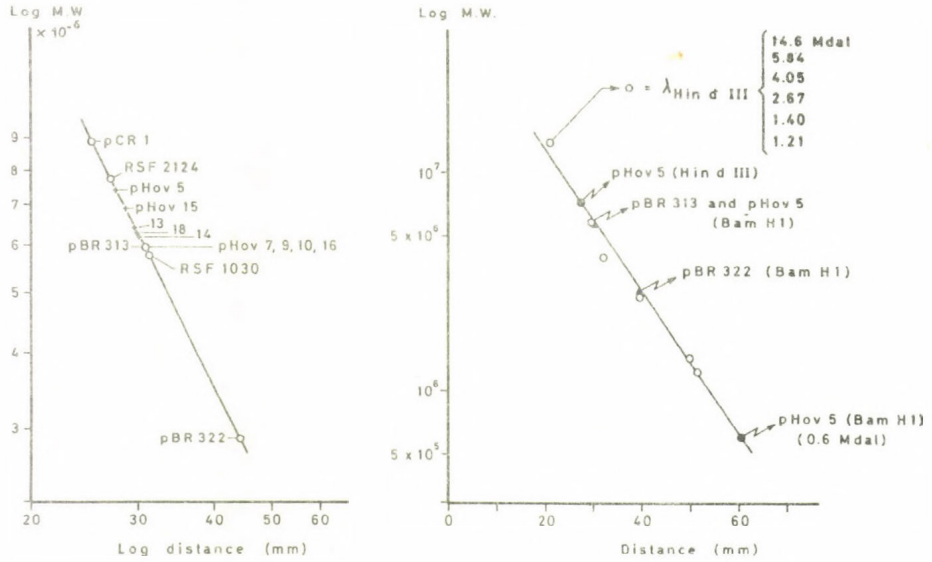

Fig. 8 Agarose gel electrophoresis of pBR 313-barley DNA recombinant plasmids. Left, analysis of supercoiled molecules. Right, analysis of pBR 322 (▲), pBR 313 (△) and p Hov 5 (●) cleaved with Bam HI, and p Hov 5 cleaved with Hind III (o).

Fig. 8 shows that most recombinant plasmids contained very small stretches of barley DNA (0.4 Mdal or less) except for the plasmid designated p Hov 5, found to contain an extra piece of DNA of about 1.2 Mdal (twice the 0.6 Mdal fragment found after Bam HI digestion of pHov 5). Fig. 9 shows the scanning of a negative picture of a 1 % agarose gel electrophoresis performed on Bam HI cleaved pBR 313 and pHov5.

Such a recombinant plasmid will be used in DNA uptake experiments with plant protoplasts in order to compare the relative rates of degradation of its bacterial and plant moieties. It is also possible that a partial homology between the molecular vector and its host DNA might facilitate integration of its heterologous part as was found in the transformation of yeast (5).

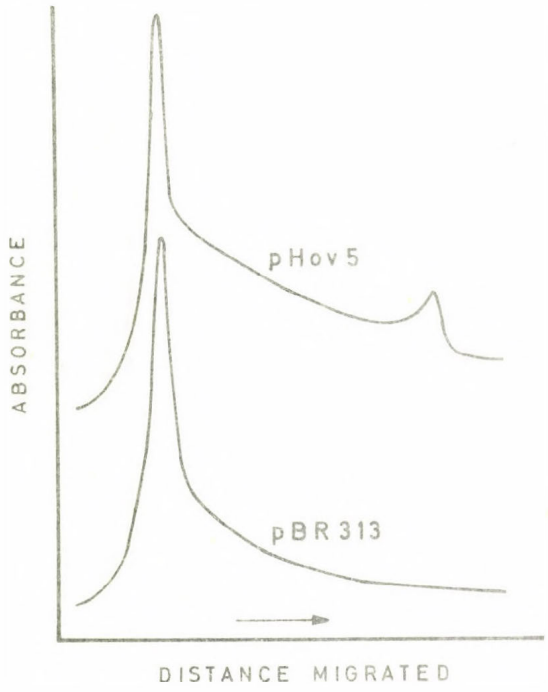

Fig. 9 Densitometer tracing of an agarose gel electrophoresis of pBR 313 and ov5 cleaved with Bam HI. Gel stained with ethidium bromide.

5. Detection of Gene Products

The biological expression of exogenous DNA taken up by protoplasts can be checked at the level of its transcription by the host DNA polymerases and at that of the synthesis and enzymatic activity of the polypeptides it codes for. Transcription is in theory easier to check for especially in the case of recombinant plasmids of low kinetic complexity. These experiments can be performed with DNA extracted from protoplasts incubated with a given plasmid DNA and subsequently labeled with (^{32}P)-ortophosphate.

The radioactive DNA can then be hybridized to the donor DNA either in solution or transferred to strips of nitrocellulose after restriction digestion and electrophoresis in agarose gels (44). Positive preliminary results using solution hybridization and fractionation of RNA : DNA hybrids on hydroxylapatite were obtained in experiments aiming at the study of the transcription of the pTi plasmid from *Agrobacterium tumefaciens* 1D 135 taken up by cowpea protoplasts (45). However, these results have to be confirmed by a more specific technique such as Southern's (44) before definitive conclusions can be made.

Two major difficulties are encountered in these experiments: (i) in the absence of selection, it is difficult to know what proportion of the protoplasts has taken up DNA and how much of

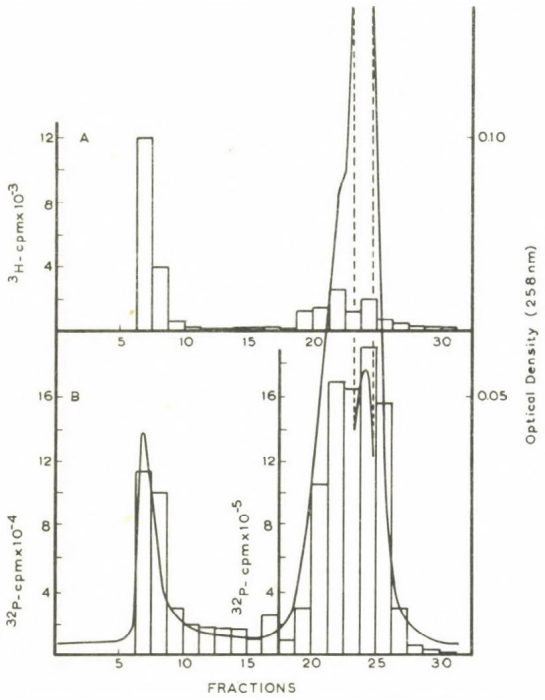

Fig. 10 Molecular sieving on Sepharose 4B of labeled nucleic acids extracted from cowpea protoplasts. A Protoplasts incubated for 16 hours with 0.1 mCi (^{3}H)-dThd in the presence of 0.01 mM FdUrd, B Protoplasts incubated for 16 hours with 1 mCi (^{32}P)-orthophosphate ——, optical density at 258 nm.

it for how long and (ii) the specific radioactivity of protoplast RNA labeled *in vivo* may be too low with respect to the amount of foreign message transcribed in the protoplasts. It is our experience that (^{3}H)-uridine is very inefficiently used by cowpea protoplasts for nucleic acids synthesis (P.F. Lurquin, S.T. Liu and C.I. Kado, unpublished). However, close to 7x10^{6} cpm can be incorporated into the RNA of a concentrated suspension of protoplasts if (^{32}P)-orthophosphate is added to the culture medium devoid of phosphate (Fig. 10).

If needed, high specific activities can be obtained by labelling *in vitro* protoplast RNA with Na(^{125}I) (46).

These problems can be overcome if the donor DNA harbors genetic markers selectable at the plant cell level. This seems to be the case of the *A. tumefaciens* pTi plasmids which appear to be involved in the ability of crown gall cells to proliferate in the abcence of hormones, to produce nopaline or octopine and whose T region is replicated and transcribed in these cells (47-49). Moreover, such transformed cells can be produced

in vitro by incubating regenerating tobacco protoplasts and virulent *A. tumefaciens* cells (50).

There are indications that such a phenomenon can also be observed by treating freshly isolated tobacco protoplasts with pTi from *A tumefaciens* B_6S_3 at high pH in the presence of $CaCl_2$ as described above. Others have reported similar observations by treating Petunia protoplasts with pTi complexed with poly-L-ornithine (51). Although lysopine dehydrogenase activity has been observed in some of these "transformed" clones, it remains to be demonstrated that at least part of the pTi DNA is present and transcribed in these clones.

CONCLUSIONS

There is good evidence that higher plant protoplasts can take up DNA (15, 26, 27, 41, 52, 56 and this study). However, the stability of the donor DNA in the medium and that of DNA bound to protoplasts critically depends on the configuation of the donor DNA and the conditions of incubation. Also, circumstantial evidence indicates that plasmid DNA from virulent *A. tumefaciens* might be expressed in protoplasts treated under conditions known to enhance DNA uptake. These observations altogether strongly suggest that the genetic manipulation of plants at the cellular level might soon become a reality.

Alternative routes to plant genetic engineering do exist. For instance, it was shown that *E. coli* Tn 7 DNA inserted within the *A. tumefaciens* pTi T37 plasmid could be detected in tobacco crown gall tumors induced by bacteria carrying this plasmid (49). Thus, this technique is theoretically applicable to a very large variety of dicotyledonous plants.

Another approach yet consists in treating dry seeds with concentrated DNA solutions, a technique leading to high uptake values (57). Furthermore, it was reported that seeds from *Arabidopsis thaliana* thiamine mutants treated with DNA harboring functional thiamine genes yielded plants seemingly corrected for their thiamine deficiency (58). Obviously, the two techniques mentioned above do not present some of the drawbacks associated with plant protoplasts such as difficulties to regenerate callus tissues or green plants. However, they possess disadvantages of their own such as the relatively low number of individuals which can be handled in one experiment and much less flexible conditions of treatment with donor genes. More work is needed to determine the actual applicability of these different approaches.

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GENETIC MODIFICATION OF PLANT CELLS BY TRANSFORMATION AND SOMATIC HYBRIDIZATION

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I. INTRODUCTION

There is general agreement that the introduction of foreign genes into the plant genome would be of importance to molecular biological studies with plant cells. If certain genes could be stably maintained and expressed, one could study, for instance, how their activity is regulated, in order to know more about the mechanisms involved in regulation of genome activity in eukaryotes. From an agronomical point of view, genetic transformation of plant cells could be used as a technique to improve plants by incorporation of certain (favourable) new characters. Until now, a reproducible procedure for transformation of plant cells has not been found. In developing systems for the genetic modification of plants, at least two approaches can, in principle, be followed.

The first is to fuse cells with different properties, using the technique of somatic cell hybridization. This allows the study of nuclear and cytoplasmic genome interactions and may lead to the creation of new species from the somatic hybrid cells (1, 2). The second approach is to make use of isolated DNA for the introduction of new genes in plant cells.

During the last few years it has become clear that the induction of the plant tumour Crown Gall by *Agrobacterium tumefaciens* is a natural example of genetic transformation (3,4,5). It is a valuable model to study, in order to determine requirements at the molecular level to transform plant cells (6). Moreover, Crown Gall cells are very useful in the study of the neoplastic condition of tumour tissue in relation to mechanisms controlling cell differentiation (7).

II. THE CROWN GALL DISEASE

1. General

The Crown Gall tumour is characterized by non-self-limiting cell proliferation. It arises when *A. tumefaciens* infects wound sites.

Only dicotyledonous plants appear to be susceptible. During infection *A. tumefaciens* cells penetrate the intercellular spaces and injured cells, from which they transform adjacent cells. The bacteria do not penetrate the plant cells which are converted to tumour cells. An attachment of the bacteria to the plant cell wall appears to be essential for tumour induction. It has been postulated that transmission of a tumour inducing principle can take place through the connection of the bacterium with the plant cell (8). On Kalanchoe stems the whole process of tumour induction is completed in 20 hours (9). Presumably, cells are transformed before they initiate cell division if this is triggered by wounding. After the tumour induction has taken place, the continued presence of viable bacteria is not required for tumour growth.

Crown Gall cells acquire a number of stably inherited new properties. The most remarkable are:

1. Phytohormone independent growth in tissue culture, whereas normal cells need these phytohormones for continuous growth.
2. The capacity to develop into a phenotypically identical tumour when grafted onto a healthy plant.
3. The capacity of many, but not all, types of Crown Galls to synthesize one of two types of unusual amino acid derivatives. One type produces octopine, octopinic acid, lysopine and histopine (10), whereas the other type produces nopaline and nopaline acid (ornaline) (11). Crown Gall cells continue the synthesis of unusual amino acid derivatives in tissue culture and even when shoots (6, 12) or complete plants are regenerated (13).

The type of unusual amino acid derivative synthesized depends on the bacterial strain used for tumour induction and is not specified by the host plant (14, 15, 16). Although some controversy existed (17, 18) it is now well documented that these compounds are tumour specific, i.e. they have not been detected in normal tissue, neither in tissue culture, nor in plants at different stages of development (15, 16, 19, 20). One tissue line, thought to have been derived from a normal callus which had become phytohormone independent in culture (habituated) also contained octopine. Recently, it was demonstrated that this tissue line was a Crown Gall line (21).

Considering the morphology of Crown Galls induced by wild type *A. tumefaciens* strains on Kalanchoe stems we observed that all strains could be subdivided in three classes (19, 22). These are those which induce tumours with a rough surface and many adventitious roots surrounding the tumour, those which give rise to tumours with a smooth surface and a number of firm roots that develop from the lower side of the tumour and those which induce small tumours with a rough surface and almost no roots. The tumours of the first type always contain octopine (octopine type tumours) and those of the second type contain nopaline (nopaline type tumours), whereas the tumours of the third type do not contain known tumour-specific amino acid derivatives (null –type tumours). It cannot yet be excluded that "null-type" tumours do contain amino acid derivatives different from the

compounds of the octopine and nopaline family. Bacteria that induce octopine tumours are able to utilize compounds of the octopine family as nitrogen- or carbon source but not nopaline. Bacteria that induce nopaline tumours can only use compounds of the nopaline family as N- or C-source. Null-type tumours are induced by bacteria which cannot catabolise compounds of the octopine or nopaline family. A few exceptional strains exist that catabolise nopaline but induce smooth type tumours without nopaline and there are avirulent *Agrobacteria* which can utilize octopine. The strict correlation between bacterium-type and tumour-type suggests that the bacteria carry genes which in a different way determine the final outcome of the transformation process. These genes are located on a large plasmid in *A. tumefaciens* cells, called the Ti-plasmid, which is responsible for oncogenicity.

2. Ti-Plasmids

Each *A. tumefaciens* cell harbours one or more large plasmids. One of these extrachromosomal DNA elements, the Ti-plasmid, carries genes which are essential for its oncogenic capacity (16, 23, 24). Large plasmids have also been detected in several non-oncogenic *A. radiobacter* strains (25) and in *Rhizobium* (26). Rhizobia are closely related to Agrobacteria and induce symbiotic nitrogen fixation on Leguminous plants.

The Ti-plasmids present in *A. tumefaciens* carry the genetic information for:

1. Oncogenicity,
2. Tumour morphology,
3. Synthesis of either octopine or nopaline in Crown Gall cells,
4. The specificity and activity of an enzyme system for octopine or nopaline utilisation,
5. A bacterium - bacterium conjugative mechanism (tra-genes).

3. T-DNA

Both oncogenicity and the synthesis of one of the unusual amino acid derivatives in Crown Gall cells are an expression of the presence of a fragment of the Ti-plasmid in these tissues(27). This fragment, which is not necessarily of the same size in different Crown Gall callus tissues and is integrated in the host genome (47), is called the T-DNA. It is not yet known whether the complete Ti-plasmid is transferred and processed in the plant cells or whether only a fragment, produced in the bacterium, is transferred to the plant cells. It is also not known whether the T-DNA is integrated in the plant cell nucleus. From cell fusion experiments, however, it is indicated that at least that part of the T-DNA, responsible for LpDH activity and hormone independent growth is located in the nucleus (7). Whether the T-DNA is integrated on a specific site or on different sites in the

genome is also an open question. It is also not known whether the T-DNA is integrated as one DNA-fragment or as a number of fragments. At least part of the T-DNA has been shown to be transcribed into RNA in tumour cells (28). Since some of this RNA contains poly A, we can conclude that T-DNA transcripts are under an eukaryotic control system and might have a mRNA function (29). Whether the RNA is also transcribed into protein is not yet known. If it is translated, possible candidates for the protein produced are the enzymes lysopine dehydrogenase (LpDH) and nopaline dehydrogenase (NpDH), which catalyse the synthesis of compounds of the octopine family and the nopaline family, respectively.

III. CROWN GALL INDUCTION

1. Crown Gall Tumours Induced *In Vivo* by *A. tumefaciens* Mutants

To establish the positions, number and character of genes on T-DNA which are responsible for integration, phytohormone autotrophy, regulation of the regeneration capacity of the tumour cells and synthesis of LpDH or NpDH, plasmid mutants are required. Therefore transposon insertion mutants and deletion mutants of *A. tumefaciens* have been used in transformation experiments *in vivo* with Kalanchoe, tomato, patunia, and tobacco as well as in transformation experiments *in vitro* with isolated tobacco protoplasts. Several mutants with transposon insertions or with deletions in or near the highly conserved region of the T-DNA have been isolated (30, 31, 32, 33). These mutants gave tumours *in vivo* with a strongly altered phenotype. Transposons are especially suitable for this purpose as there are methods to select for their presence in the plasmid. Transposons inactivate the gene into which they are inserted and can have a polar effect on neighbouring genes. Properties of some of the mutant strains are summarized in Table 1.

The mutations in the three strains LBA 4018, LBA 4210 and LBA 4060 had occurred in a DNA region that is highly conserved in all three types of Ti-plasmids and which is partially or completely included in the T-DNA (48) (Fig. 1). The mutant LBA 4210 induced both on stems of *Kalanchoe diagremontiana* and *Nicotiana tabacum* cv. Petit Havana tumours with excessive root formation at the base of the tumour. The mutant LBA 4060 induced on stems of both these plants tumours with teratomata formation. These teratomata, in the case of Kalanochoe, arose above the tumour, and in the case of the tobacco, arose on the upper part of the tumour. (Fig. 2) Preliminary experiments (32) in which Kalanchoe stems were infected with a mixture of both the LBA 4210 and LBA 4060 have resulted in tumour formation comparable with a tumour induced by the wild type *A. tumefaciens* (32). This suggests that complementation of the insertion mutations present in these bacterial strains occurs leading to the development of a "wild type" tumour. The results obtained are indicative of an active role for the Ti-plasmid DNA in the control of phytohormone levels in the plant tumour cells. It is well known that the development of roots or shoots by plant tissue in culture is re-

TABLE 1 Characteristics of *Agrobacterium tumefaciens strains used in transformation of N. tabacum protoplasts in vitro*

Strain	Plasmid	Virulence *in vitro* on protoplasts	Amino acid derivative in tumour	Tumour morphology				Ref
				On *N. tabacum* sR1 protoplasts	On *N. tabacum* sR1 stems	On Kalanchoe stems	On *N. tabacum* White Burley stems	
LBA 4011	none	-	-	-	-	-	-	(33)
LBA 4001	wild type Ach-5	+	octopine	oct.tumour	oct.tumour	oct. tumour	oct. tumour	(45)
LBA 4013	pAL 102 from Ach-5	+	octopine	oct.tumour with teratomata	oct. tumour	oct. tumour	oct. tumour	(45)
LBA 4058	pAL 8374 from T37	+	nopaline	nop.tumour with teratomata	nop. tumour with teratomata	nop. tumour	not tested	(22)
LBA 4057	pAL 672 cointegrate of oct. and nop.plasmid	+	octopine +nopaline	nop.tumour with teratomata	nop. tumour with teratomata	nop. tumour	not tested	(22)
LBA 4060	pAL 108 insertion of left part of T-DNA	+	octopine	oct.tumour	oct. tumour with teratomata	oct. tumour with teratomata	small oct. tumour	(45, 32)
LBA 4210	pAL 288 insertion of middle part of T-DNA	+	octopine	oct.tumour with roots	oct. tumour with excessive roots	oct. tumour with excessive roots	small oct. tumour	(31, 45)
LBA 4018	pAL 105 deletion of right part of T-DNA	±	-	not tested	no tumour	small oct. tumour	no tumour	(30, 45)

gulated by the balance between auxins and cytokinins in the cells. Tumours induced by LBA 4210 are expected to contain a relatively high level of auxin-like hormones, resulting in extensive root formation, whereas tumours induced by LBA 4060 presumably contain a high level of cytokinins, resulting in shoot formation. A more detailed description of these observations will be published elsewhere (32).

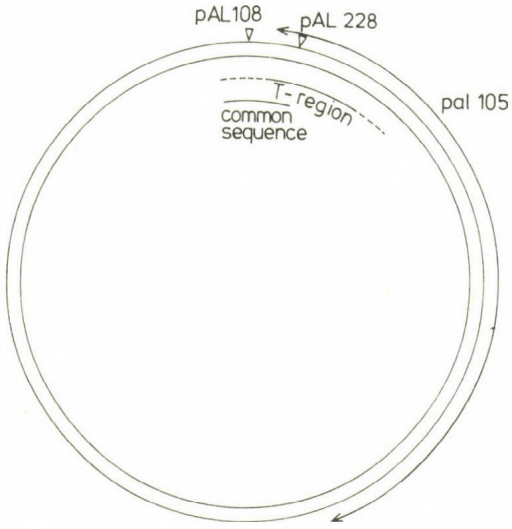

Fig. 1 Schematic representation of the octopine Ti-plasmid

The pAL numbers represent the position of the insertions (▽, pAL 108 and 228) and the deletion (⟷, pAL 105). The dotted lines indicate that the accurate endpoints of the T-region and the common sequence are not known (48).

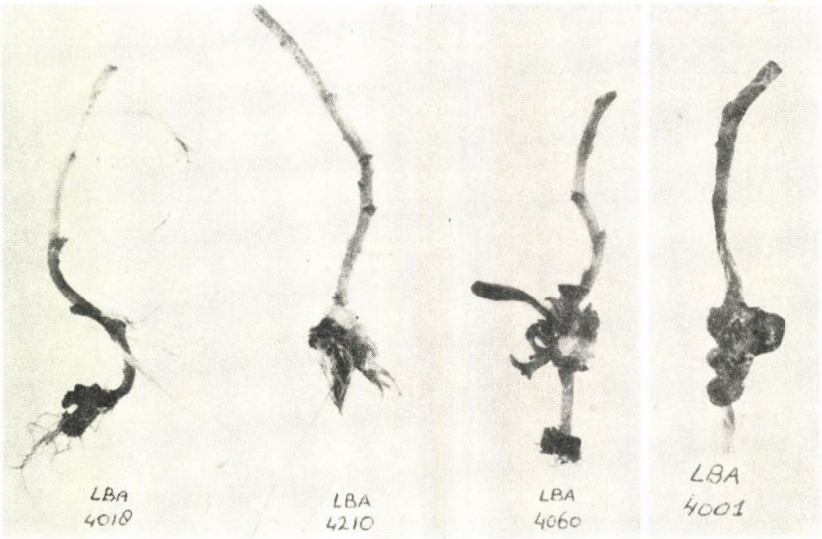

Fig. 2 Induction of tumours on *N. tabacum* cv. Petit Havana (SR1) by some *A.tumefaciens* mutants after 6 weeks.

2. Transformation of Tobacco Protoplasts by *A. tumefaciens*

Crown Gall induction *in vivo* is the result of transformation of plant cells by virulent *A. tumefaciens*. Wounding of the plant has been found to be essential for tumour formation. This causes dedifferentiation of cells, after which transformation can occur leading to unlimited cell proliferation and the development of a plant tumour at the site of infection. The development of a procedure to achieve *in vitro* tumour induction using isolated protoplasts will help to elucidate the initial processes that take place during tumour induction and development. It also may help to clarify the actual role of the T-DNA using *A. tumefaciens* Ti-plasmid mutants in *in vitro* transformation experiments.

The procedure described here involves the incubation of three-day-old protoplasts from a streptomycin resistant strain of *N. tabacum* (SR1) with different strains of *A. tumefaciens*. Selection of transformed cells against normal SR1 cells was based on the hormone independent growth of Crown Gall cells and on the streptomycin resistance of the SR1 cells. Details of the procedures for transformation, selection and characterization of transformants are described elsewhere (6).

The results of transformation with different virulent and avirulent bacterial strains is also shown in Table 1. It can be concluded that transformation of the cells occurs, both with octopine type and with nopaline type *A. tumefaciens*. The tumour specific enzymes LpDH or NpDH are present in respectively 60 and 10 % of the isolated colonies.

TABLE 2 Frequency of transformation following incubation of *Nicotiana tabacum* SR1 protoplasts with a virulent strain of *Agrobacterium tumefaciens*

Bacteria/10^5 protoplasts	Plates with colonies/Plates* seeded	Number of colonies	Frequency of colonies
0	0/4	0	0
3×10^5	0/4	0	0
6×10^5	1/4	1	25×10^{-6}
12.5×10^5	4/4	20	5×10^{-4}
25×10^5	4/4	50	12.5×10^{-4}
50×10^5	4/4	c. 400	1×10^{-2}
100×10^5	4/4	c. 400	1×10^{-2}

* One plate was seeded with 10^4 cell clumps.

The frequency of transformation depends on the bacterial strain (6) and on the ratio between bacteria and SR1 cells as is shown in Table 2. It was found that the transformation frequency increased upto a ratio of bacteria to plant cells of 50 to 1. At this point a saturation level is reached.

An accurate estimation of the transformation frequency is hampered by the observation that, after addition of the bacteria to the cells, the cells agglutinate within a period of approximately five hours, depending on the bacterial strain. This agglutination may be the result of attachment of the bacteria to the plant cells. Attachment of *A. tumefaciens* to plant cells is described by a number of authors (34, 35) and is also shown in Fig. 3.

Tobacco cells agglutinate more quickly when incubated with virulent Ti-plasmid containing *A. tumefaciens* strains than when incubated with avirulent strains. On the other hand, normal tobacco cells agglutinate more than Crown Gall cells when incubated with a virulent strain. It is clear that both the bacterial strain and the plant cell determine whether and how much agglutination takes place.

The observation of a saturation level in the transformation frequency may be explained by the proposal that for transformation of single cells, attachment of the bacterium to the plant cells is a prerequisite. Taking into account that the majority of the bacteria do not take part in the transformation process, because they also clump and attach to dead cells and cell debris, the effective attachment is presumably limited to a certain number of specific attachment sites on the surface of the plant cells. When all these sites are occupied, the saturation level is reached. Although the attachment of the bacteria to

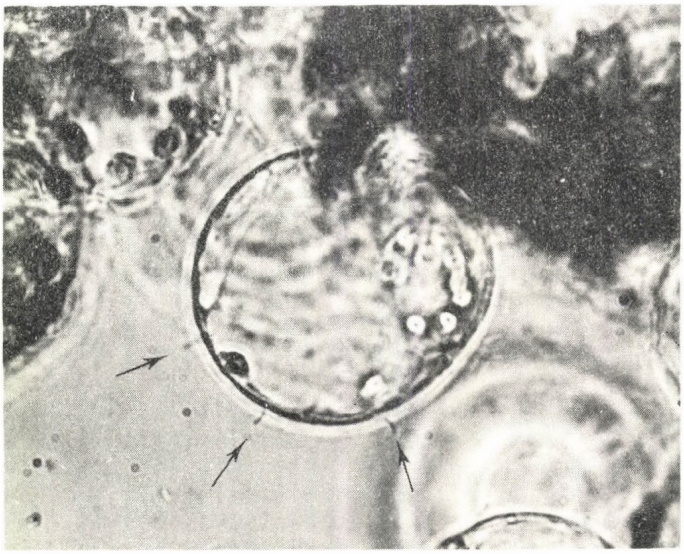

Fig. 3 Phase-contrast photomicrograph of normal *N. tabacum* SR1 cells (10^5 cells per ml) and *A. tumefaciens* strain LBA 4013 (10^7 per ml) after incubation together in culture for 5 hours. Attachment of the bacteria to the cell wall is indicated by arrows.

the cells, resulting in clumping of the cells, is an interesting aspect of the initial process of tumour induction, it hampers the efforts to transform single cells and to follow the single cells in their development to pure transformed clones.

As shown in Table 1, transformation *in vitro* has been obtained with bacterial strains with an octopine plasmid and with a strain containing a nopaline plasmid. Hormone independent calli have also been isolated following transformation with a bacterial strain in which a co-integrate plasmid, consisting of an octopine and a nopaline plasmid, is present (LBA 4057) (22). In Crown Gall tumours, induced *in vivo* on a tobacco plant with LBA 4057, the enzyme NpDH, catalyzing the synthesis of nopaline, was detected immediately in callus tissue obtained after culture of tumour tissue, whereas the enzyme LpDH was only found after two subcultures (two months). However, *in vitro* transformation with LBA 4057 has yielded transformants in which only the enzyme NpDH has been found so far. Whether also the enzyme LpDH will be expressed has to be seen after another two months in tissue culture. Whether the LBA 4057 tumours consist of a mixed population of octopine cells and nopaline cells, or whether they consist of cells in which fragments of both plasmids are present is not yet known. This will be investigated by cloning the tumours through isolation and culture of protoplasts.

From transformation experiments with the octopine bacterium LBA 4001 two colonies have been obtained which have lost their hormone indepedendent character after two subcultures on hormone--free medium. In these calli, which are now kept on a hormone containing medium, the enzyme LpDH is still present. From the transformation experiments, colonies have also been isolated on phytohormone-free medium in which no LpDH could be detected. After a few passages, these calli had to be cultured on hormone containing medium. Concerning the properties described here, these tissues now behave like control SR1 callus tissues.

A possible explanation for the loss of hormone independent growth whereas the LpDH activity remains present during subculture is segregation or complete loss of T-DNA genes. A similar loss of tumour markers has not been observed in tumour tissues obtained on SR1 plants after tumour induction *in vivo* with the same bacterial strains.

Callus tissues, obtained after transformation with LBA 4013 and with LBA 4058 developed shoots on hormone-free culture medium. The shoots are characterized by an extensive formation of axillary shoots, by the absence of roots and by the presence of the tumour specific enzyme LpDH or NpDH, respectively.

3. Transformation of Tobacco Protoplasts by Isolated Ti-Plasmid DNA from *A. tumefaciens*

Although it is possible to transform plant cells by incubation with bacteria there is always the problem, with this procedure, of killing the bacteria afterwards. On the other hand, bacterial induced transformation gives no answer to the question whether Ti-plasmid DNA alone is sufficient to induce tumours. In the plasmid transformation experiments *N. tabacum* SR1 protoplasts were used as recipients. Table 3 gives a summary of the different treatments and the results obtained.

Only the Ca/high pH method has given a positive result. This method was originally used by Keller and Melchers (37) for protoplast fusion. In the transformation experiments, it involved the incubation of about 10^6 protoplasts in 3 ml Ca/high pH solution containing 10 ug/ml DNA. After the incubation, the protoplasts were washed and cultured in hormone containing medium. The hormone concentrations used were different from those required for culture of normal SR1 protoplasts (38). When normal SR1 cells are grown on these hormone concentrations they do divide, but upon transfer to hormone-free medium division immediately stops. Cells transformed by DNA continue their growth under the hormone-free conditions.

From one such experiment , using the Ca/high pH method and Ti--DNA from a virulent strain of *A. tumefaciens* 7,colonies were isolated on hormone-free medium (Table 3). These colonies grew well on hormone-free medium and two of them contained the tumour specific enzyme LpDH.

TABLE 3 Crown Gall transformation of tobacco protoplasts by Ti-plasmid DNA

Induction of uptake*	Source of DNA	Number of colonies Screenedx10^5	Hormone independent	LpDH$^+$
Poly-L-ornithine +Zn^{++}	Ti-plasmid	3	-	-
	none	2	-	-
Zn^{++} + high pH	Ti-plasmid	1.2	-	-
	none	1.2	-	-
Ca^{++}-coprecipitated DNA	Ti-plasmid	5	-	-
	SS**	2	-	-
	none	1.2	-	-
0.1 SSC in K3 medium	Ti-plasmid	1.4	-	-
	none	1.4	-	-
Ca^{++} + high pH	Ti-plasmid	10	7	2
	SS	10	-	-
	none	2	-	-

* Details on the treatments will be published (46).

** SS: salmon sperm DNA.

Since spontaneous regeneration of these calli did not occur attemps were made to regenerate shoots from the two LpDH positive calli by culturing them on phytohormone-containing medium (0.5 mg/l benzyladenine, 2 mg/l IAA). Under these conditions, regeneration of normal SR1 derived callus was easily achieved. However, the shoots that arose on the transformed calli showed an SR1-like phenotype. They developed roots and were LpDH negative. Since shoots obtained spontaneously from transformed calli obtained from bacteria-treated SR1 cells did contain LpDH and were unable to develop roots, we thought it likely that the shoots from the Ti-plasmid transformed calli arose from normal cells present in the transformed callus. Due to aggregation of normal and transformed cells, the transformed calli could be a mixture of these cells. These normal cells can divide on hormone-free medium due to the presence of growth hormones produced by the real transformed cells (39). Another explanation for these shoots is, that they have been induced from transformed cells that have lost their tumour markers as a result of segregation during subcultures. Segregation can occur and has also been observed with bacterial transformed calli. Some of these calli are unable to form shoots spontaneously, whereas normal SR1 shoots can be isolated after culturing on the hormone containing medium.

Transformation of protoplasts by isolated Ti-plasmid DNA from *A. tumefaciens* has also been obtained by Davey and co-workers (40).

They used *A. tumefaciens* Ti-plasmid DNA and poly-L-ornithine to transform petunia protoplasts. From one experiment LpDH positive petunia transformants have been isolated.

Experiments are in progress to find out more about the reproducibility of the DNA transformation of protoplasts and the transformation frequency. At this moment, further studies are also in progress in our laboratory to look for the presence of Ti-plasmid fragments in the DNA isolated from the transformed tissues and to increase the DNA transformation frequency to a level at which this technique would be suitable for genetic engineering in plants.

4. Fusion of Normal Cells with Crown Gall Cells

Somatic cell hybridization between tobacco Crown Gall protoplasts and protoplasts from normal tobacco leaves has been carried out in order to study the expression of the Crown Gall characters in the presence of a normal genome. For this purpose we used a cloned octopine producing tobacco cv White Burley Crown Gall callus (B6S3) which could not be induced to form shoots in tissue culture. Protoplasts from this tissue were fused with protoplasts isolated from leaves of the tobacco mutant SR1, which is streptomycin resistant. Selection of somatic hybrid cells was based on the properties of both parental cells as they are summarized in Table 4.

TABLE 4 Properties of *N. tabacum* parental cells and their hybrids

Properties	*N. tabacum* SR1	*N. tabacum* B6S3	SR1 + B6S3 hybrids
Phytohormone independence	-	+	+
Streptomycin resistance	+	-	+
Greening of callus	+	-	+
Regeneration	+	-	+
LpDH activity	-	+	+

The normal tobacco SR1 protoplasts are resistant to streptomycin (1 mg/ml), whereas the Crown Gall protoplasts are sensitive to the drug in this concentration. This allowed a first selection step for streptomycin resistant cells in a mixture of B6S3 Crown Gall protoplasts, SR1 protoplasts and presumed hybrid cells which were plated in a liquid culture medium supplemented with 1 mg/ml streptomycin, 0.1 mg/l auxin and 0.2 mg/l cytokinin. These conditions permitted growth of unfused SR1 or homokaryon SR1 cells and of B6S3 + SR1 fusion products. The small colonies

that developed were cultured for a second selection step on solidified agar, streptomycin containing medium without phytohormones. After this step, only a few cell colonies were able to continue their growth. These colonies have been isolated and subcultured on hormone-free medium for characterization of the phenotypic traits. Details of these fusion experiments will be published elsewhere (7).

The expression of the tumour markers, such as the presence of the tumour specific enzyme LpDH and the capacity of the cells for hormone independent growth has been studied in relation to the regenerative potential of the hybrids.

The physiological characters of 4 out of 16 independent colonies obtained indicate that these are real hybrids. These properties are summarized in Table 4. The combination of greening, regeneration and streptomycin resistance on the one hand, and the presence of the enzyme LpDH on the other hand, mark these callus tissues as true hybrids. The 12 other calli have only the streptomycin resistance that makes them distinguishable from the parental B6S3 callus. It cannot be excluded that B6S3 cells have overcome the streptomycin selection step although this has not been found in control experiments.

The development of shoots from hybrid callus tissues indicates that it is not necessary for both fusion partners to be able to regenerate *in vitro* in order to obtain regeneration of hybrids. Comparable results have been published for fusion between normal cells of *Petunia* (41), *Nicotiana* (42) and *Datura* (43). The ability to form shoots is a SR1 property. However, when tumours are formed on SR1 by infection with *A. tumefaciens* strain B6S3, the regeneration capacity of the obtained tumour cells is suppressed. Shoot development from the hybrid tumours shows that the regeneration capacity is restored by the presence of a normal SR1 genome in the hybrids and also demonstrates that the ability to regenerate shoots is a dominant trait.

5. Shoots Obtained After *In Vitro* Tumour Induction

Shoots have been isolated from tumour tissues obtained *in vitro*, either after cell fusion or after bacterial-induced transformation of protoplasts. The properties of these shoots are shown in Table 5.

TABLE 5 Properties of hybrid and transformed SR1-shoots

- No root formation,
- Thick stems and leaves
  small size growth
  formation of lateral shoots
- LpDH positive
- Resistant against infection with
  virulent strains of *Agrobacterium*

An important property is the presence of the enzyme LpDH in shoots both from the hybrids and from tumours induced by octopine strains of *A. tumefaciens*, whereas shoots from nopaline tumours contain the enzyme NpDH. The presence of these enzymes is always correlated with an abnormal morphology, with the absence of roots and with the absence of apical dominance. These features can best be explained by assuming that the balance of the hormones, auxin and cytokinin, is abnormal in the shoots. Both the axillary shoot development and the absence of root formation may be due to an overproduction of cytokinin. The fact that not all LpDH positive tumours regenerate shoots suggests that the genes responsible for LpDH activity and those responsible for maintenance of neoplastic growth can be expressed differentially. The formation of shoots shows that the induction of neoplastic growth is not an irreversible process. However, it is not known why some tumours develop shoots and others do not under the same circumstances. A third character of the tumour tissues, the phytohormone independent growth can be present independently of the LpDH activity and the regeneration capacity. This is shown by the observation that LpDH negative transformants have been isolated which are phytohormone independent, and LpDH positive, which are phytohormone dependent for their growth. In conclusion, it can be said that the synthesis of LpDH, the maintenance of undifferentiated growth and the production of phytohormones can be expressed differentially. It should be mentioned, however, that the expression of these phenotypic traits can be regulated by the presence or absence of phytohormones in the culture medium. Table 6 shows the effect of hormones on tumour tissues and on normal tissues.

TABLE 6 Influence of phytohormones on the growth of protoplasts from normal and tumorous tissues

Origin of protoplasts	hormones[1]	growth	hormones[2]	growth	hormones[3]	growth	hormones[4]	growth
B6S3 callus	-	callus	-	callus	-	callus	-	callus
SR1 shoots	+	callus	+	callus	+	callus	+	callus
	+	callus	-	none				
Hybrid SR1 shoots	+	callus	+	callus	+	callus	+	callus
Transformed SR1 shoots	+	callus	-	callus	-	callus	-	shoots
	-	none						

Protoplasts isolated from hybrid or transformed shoots require exogeneous supplied phytohormones to initiate cell division. After callus tissues of sufficient size have been formed, all colonies can be subcultured indefinitely on a hormone-free me-

dium, whereas the LpDH activity is present in all individual subclones. These observations indicate that the genetic information for these tumour characters is present in all individual cells of the shoots. That the expression of neoplastic growth versus regeneration can also be regulated by exogeneous phytohormones is demonstrated in Fig. 4.

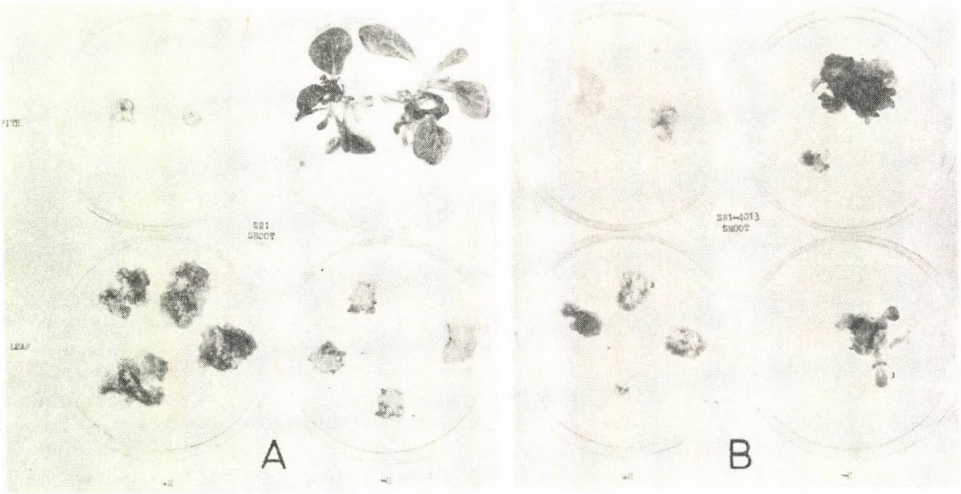

Fig. 4 Representation of the growth response of pith and leaf explants from a normal *N. tabacum* SR1 shoot (A) and a transformed SR1 shoot (B) in the presence or absence of phytohormones in the culture medium. The explants have been cultured for one month. The hormones were auxin (3 mg per l NAA; 0.1 mg per l 2,4-D) and cytokinin (0.04 per l kinetin).

When fragments of leaves and pith of the tumorous shoots were placed on a hormone-free and a hormone containing medium, shoots developed from both types of explants without callus formation on the hormone free medium. Both types of explants, however, developed fast growing callus tissues on hormone containing medium. The enzyme LpDH was present in the tissues grown from both types of explants on both media. Both pith and leaf explants from normal SR1 shoots, cultured on hormone containing medium, developed fast growing callus tissue. The pith explant, cultured on hormone-free medium gave rise to shoot regeneration, whereas the leaf explants failed to grow. These experiments show that the tumorous callus tissue and shoots do not show a permanent and irreversible phenotype. A reversal of the unorganized growth, leading to a more or less normalization of the differentiation, has been demonstrated. Whether organized growth or unorganized growth will occur depends on how the genetic information in the tumour cell is regulated by the phytohormones. Evidence for the suppression of the neoplastic condition has also been obtained

by Braun (36). He used teratoma-tumour tissues of single cell origin which were derived from nopaline-producing tobacco Crown Gall tissues. After application of certain manipulations morphological and functionally normal shoots were obtained that flowered and set seed. It was shown that the tumour shoots still synthesized NpDH and contained T-DNA (44). However, haploid tissues derived from anthers and plants of the F1 generation lacked both NpDH and T-DNA (44). The loss of T-DNA may have taken place during meiosis.

IV. THE POTENTIALS OF THE SYSTEM

Since *A. tumefaciens* is able to transform plant cells of different species, both *in vivo* and *in vitro*, the bacterium itself, carrying a modified Ti-plasmid, might be a good tool for genetic engineering of plant cells. From a technical point of view it may be concluded that the first steps on the long road leading to successful genetic engineering of plants through the integration of new genes have been taken. What types of new genes will be incorporated, will be determined by the possibilities for manipulation of the *A. tumefaciens* Ti-plasmid. It is clear that Ti-plasmids can be transferred not only between various *A. tumefaciens* strains, but also from *A. tumefaciens* to a nitrogen fixing *Rhizobium trifolii* and *vice versa* (49). The *Rhizobium* strain, carrying the Ti-plasmid, is oncogenic on several plants and is still able to form normal, nitrogen fixing nodules on its host. If we could engineer the Ti-plasmid in such a way that foreign genes, for instance, the nif genes of *Rhizobium*, replace that part of the T-DNA which is not essential for transformation we might expect that these foreign genes can be introduced and be brought to expression in plant cells. In addition, other components involved in nitrogen fixation, like leghemoglobin, protecting the nitrogenase enzyme complex from oxidation, are also required to be present in the transformed tissue. The first problem that has to be solved, before transfer of desirable genes can be successful, is to modify the recent methods for transformation in such a way that normal, fertile plants can be regenerated from the transformed tissue in which the genetic information for foreign genes, like LpDH, is still present and should be passed on to the offspring of such plants. The problem of the absence of normal regeneration of transformed tissue can be overcome by fusing protoplasts from this tissue with normal regenerating mesophyll protoplasts of plants from the same species.

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THE USE OF PLANT PROTOPLASTS FOR TRANSFORMATION BY *AGROBACTERIUM* AND ISOLATED PLASMIDS

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SUMMARY.
Isolated *Agrobacterium* plasmids will transform higher plant protoplasts, while intact bacteria will transform regenerated cells. Transformed tissues are hormone independent, multiply when grafted onto the host plant, synthesise octopine, and show lysopine dehydrogenase activity. These are characteristic features of crown gall tumours. These results are discussed with reference to the use of *Agrobacterium* plasmids in the genetic engineering of plants.

INTRODUCTION.
Crown gall disease of dicotyledonous and gymnospermous plants is caused by virulent strains of the Gram-negative bacterium *Agrobacterium tumefaciens* carrying large (M.Wt. 96-156 X 10^6) tumour-inducing (Ti) plasmids (1-3). During transformation, the plasmid is transferred to the recipient cell, and a segment (T-DNA) is integrated into the plant DNA (4,5), where it is transcribed (6). The T-DNA can be extensive and can code for several functions, including tumourigenesis and the synthesis of opines of the octopine or nopaline type by the transformed cells. Such amino-acids are specific to the strain of *Agrobacterium*, are only present in crown gall tissues, and are utilised as carbon and nitrogen source by the micro-organism. Transformed cells can be freed of the inducing bacterium, and will proliferate autonomously in tissue culture in the absence of hormones (7).

The Ti plasmid is a natural vector which promotes the transfer, integration and expression of foreign DNA in plants. On this basis it may be possible to utilise the plasmid, or smaller T-DNA carrying plasmids, in genetic engineering. Initially this requires demonstration that cultured plant cells can be transformed to the tumourous condition by either intact *Agrobacterium* or by isolated Ti plasmids (8,9). Enzymatically isolated protoplasts provide a useful experimental system for transformation studies, since they develop into colonies of single cell origin. This paper reports the transformation of cells regenerated from wild-type *Nicotiana* leaf mesophyll protoplasts by intact *Agrobacterium*, and amplifies an earlier report of the transformation of *Petunia* suspension cell protoplasts by isolated *Agrobacterium* plasmids (10).

MATERIALS AND METHODS.
I. Protoplast isolation - Leaf mesophyll protoplasts were isolated from

Nicotiana tabacum cv Xanthi (11). Suspension cultures initiated from leaf explants of *Petunia hybrida X P. parodii* were maintained in medium (12) with 2.0 mg/l 2,4-D and 0.25 mg/l kinetin. Protoplasts were isolated using 2% w/v Driselase, 2% Meicelase (Meiji Seika Kaisha,Ltd., Tokyo), and 0.4% Macerozyme (Kinki Yakult Ltd., Nishinomiya, Japan).

2. Maintenance of *Agrobacterium* - *A. tumefaciens strain ACH-5* was cultured (28°) on a minimal medium (K_2HPO_4 10.25, KH_2PO_4 7.25, NaCl 0.15, $MgSO_4 7H_2O$ 0.5, $CaCl_2 2H_2O$ 0.01, glucose 5.0 g/l; $FeSO_4 7H_2O$ 2.5, octopine 8.0 mg/l; Bacto-Difco agar 1.5 % w/v).

3. Culture of *Nicotiana* mesophyll protoplasts and inoculation of regenerated cells with *Agrobacterium* - Mesophyll protoplasts were cultured (25°, 700 lux) in 3 ml liquid over 12 ml agar solidified medium (13) containing 2.0 mg/l NAA and 0.5 mg/l BAP with 9% w/v mannitol. Protoplast numbers in the liquid layer were adjusted to give an overall plating density of 2.0×10^5/ml. Cells regenerated from protoplasts were transferred to medium with 7% w/v mannitol at day 7, and inoculated with 0.4 ml of a 24 h culture (Lab Lemco 10.0,peptone 10.0, NaCl 5.0 g/l, pH 7.0) of *Agrobacterium* at day 14. The bacterial density was 5.0×10^7/ml and 0.8×10^2/ *Nicotiana* cell (cell density was 6.4×10^5/ml). Cells were incubated with bacteria for 36 h. Uninoculated controls were also set up.

4. Selection of transformed *Nicotiana* cells - Cells were transferred to medium (liquid over agar) with 1.0 mg/ml carbenicillin (Pyopen, Beecham Research Laboratories) for 4 wk, the mannitol level being reduced to 3.5 % and finally removed during this period. Cells were harvested from the liquid layer and treated with lysozyme (10 mg lysozyme in 8.0 mM Tris HCl 0.74 mM EDTA, pH 8.0, 28°, 2h) to remove remaining bacteria by protoplasting, and plated in agar medium containing hormones (2.0 mg/l NAA and 0.5 mg/l BAP) with carbenicillin (0.5 mg/ml). The contents of each origional dish were plated as 4, 15 ml aliquots. After a further 4 wk individual colonies were transferred to agar medium (13) lacking hormones, approx 1300 colonies being sub-cultured from each of the 4, 15 ml aliquots. Colonies which continued to proliferate were selected and maintained on medium lacking hormones. Carbenicillin was omitted when the tissues were sterile.

5. Isolation of Ti plasmids and incubation with *Petunia* protoplasts - Plasmid was prepared from a 48 h *Agrobacterium* culture, purified, and ethanol precipitated by a method similar to that described (1). The DNA was pelletted (10,000 rpm, Beckman SW41, 1h, 4°), the supernatant removed, and the pellet drained. 30 µg plasmid was resuspended in 2.4 ml sterile 0.05M Tris HCl 2mM EDTA pH 8.0 containing 9% mannitol (overnight, 4°). Sterility of the plasmid was checked by plating aliquots onto nutrient agar and minimal medium with octopine . The plasmid was sterile and free from contamination by cells of *ACH-5* or other micro-organisms. It contained 60% supercoiled, 10% open circular and 30% linear DNA as judged by electron microscopy. 4×10^6 *Petunia* protoplasts were transferred to a 12 X 100 mm screw capped tube and centrifuged (90 X g, 2m). The supernatant was removed, and protoplasts resuspended in 1.0 ml 0.05M Tris HCl 2mM EDTA pH8.0 containing 9% mannitol, 10 µg Ti plasmid, and 2 µg poly-L-ornithine HBr (M.Wt. 166,000, Sigma, 1 h, 27°; 14). Controls without plasmid were also set up. Protoplasts were washed with 0.05M Tris HCl 2mM EDTA pH 8.0 containing 9% mannitol (3 X 8 ml changes), and plated at 2.0×10^5/ml in liquid medium (13) with 2.0 mg/l NAA and 0.5 mg/l BAP (25°, 700 lux).

6. Selection of transformed *Petunia* cells.- 14 days after treatment with Ti plasmid, *Petunia* cells were separated by gentle agitation, and spread

over the surface of agar medium with reduced hormones (0.44 mg/l NAA, 0.11 mg/l BAP). Cells were maintained on medium with reduced hormones for 4 mth, the osmotic pressure of the medium being reduced during the first 6 wk. Cell colonies were subsequently transferred to agar medium lacking hormones. Those which continued to proliferate were sub-cultured to hormone-free medium with regular transfer every 4 wk.

7. Induction of authentic crown gall tissues - Shoots of greenhouse-grown plants were surface sterilised (10% v/v "Domestos" bleach, 20m; 6 changes of sterile water) and cut into 5 cm lengths. Stem bases were inserted into agar medium (13) with 0.1 mg/l NAA to encourage rooting. Stem tips were removed, and the cut surface inoculated with a loopful of a log phase culture of *Agrobacterium*. Terminal tumours which developed on incubation (25°, 1,000 lux) were excised after 4 wk and maintained on hormone-free agar medium (13) with 1.0 mg/ml carbenicillin. The antibiotic was omitted when the tissues were sterile (3-4 mth).

8. Grafting of tissues selected from plasmid-treated protoplasts and authentic tumours onto host plants - Shoots of *Petunia hybrida X P. parodii* were prepared as in 7 above, and a wedge-shaped piece of tissue (40 mg) inserted into a 0.5 cm slit on each stem. Grafts were covered with sterile parafilm.

9. Electrophoretic detection of octopine in transformed tissues - 200 mg of tissue was incubated in 2 ml hormone-free liquid medium (13) containing 100mM L-arginine HCl (25°, 48h, 120 rpm). Tissues were rinsed with sterile water, blotted dry, homogenised, and centrifuged (12,000 rpm, 2m). The supernatants were retained, and 5 µl aliquots spotted 3.5 cm from the anode of a 17 X 15 cm sheet of Whatman 3MM paper, 1.0 cm apart, and dried. 4 µl of an arginine/octopine mixture (each at 0.5 mg/ml) was used as standard, together with 1 µl of ethanolic methyl green (migrates just behind the fastest compound, arginine). Electrophoresis was carried out in formic acid : acetic acid : water (5:15:80) (30m, 360V 26mA at start; 300V,33mA at finish). The stain reagent was a fresh mixture of 2 mg phenanthrenequinone in 10 ml ethanol and 1 g NaOH in 10 ml 60% ethanol. After dipping and drying, the electropherogram was observed under UV (366 nm). Authentic octopine had a yellowish-green fluorescence.

10. Detection of lysopine dehydrogenase (EC 1.5.1) activity in transformed tissues - A micro-scale method was used as described (15).

RESULTS.

1. *Nicotiana* cells incubated with *Agrobacterium* - Cell colonies present in *Nicotiana* protoplast cultures became aggregated following inoculation with *Agrobacterium*. In a typical experiment, 1300 colonies were transferred to hormone-free medium during the selection procedure. 28 of these (approx. 1 in every 2×10^4 protoplasts origionally plated, or 1 in every 8.6×10^4 cells regenerated from protoplasts at the time of inoculation) continued to grow in the absence of hormones (Fig. 1), and of 17 such colonies examined to date, 14 contained octopine (Fig. 2) and showed lysopine dehydrogenase activity (Fig. 3), the enzyme involved in the production of octopine from arginine in crown gall tumours (24). The remaining colonies did not show octopine or enzyme activity, indicating habituated tissues. Octopine and lysopine dehydrogenase were also absent from non-transformed *Nicotiana* callus. Octopine-positive tissues selected from *Agrobacterium*-treated cells showed more octopine as judged by the intensity of staining following electrophoresis of standard tissue extracts than some uncloned authentic *Nicotiana* crown gall tumours. Morphologically, the selected tissues resembled authentic *Nicotiana*

tumours, being composed of a mixture of green and colourless cells.

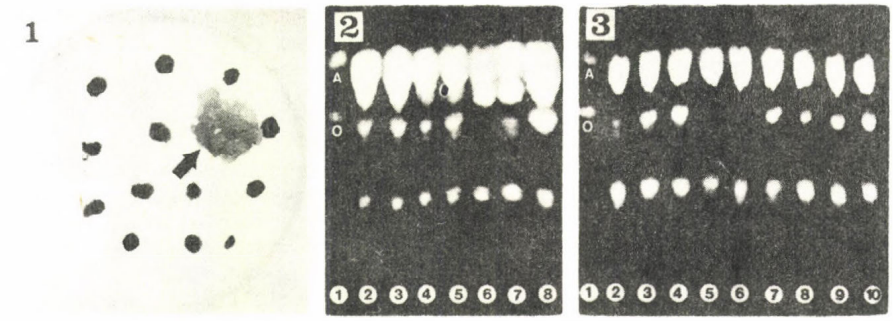

Fig.1. Transformed *Nicotiana* tissue (arrowed) selected on hormone-free medium.

Fig.2. Separation of amino-acids in extracts of tissues from *Agrobacterium*-treated cells, non-transformed callus and *Nicotiana* crown gall. Channel 1, standards, arginine (A), octopine (O); 2,3,4, tissues from *Agrobacterium*-treated cells; 5, tissue from *Agrobacterium*-treated cells + octopine; 6, non-transformed *Nicotiana* callus; 7, non-transformed *Nicotiana* callus + octopine; 8, *Nicotiana* crown gall induced by *A. tumefaciens ACH-5* bacteria.

Fig.3. Lysopine dehydrogenase activity in extracts of tissues from *Agrobacterium*-treated cells and *Nicotiana* crown gall. Channel 1, standards, arginine (A), octopine (O); 2,3,4, tissue from *Agrobacterium*-treated cells at start (2), after incubation (3), after incubation + octopine (4); 5,6,7, tissue from non-transformed *Nicotiana* callus at start (5), after incubation (6), after incubation + octopine (7); 8,9,10, *Nicotiana* crown gall at start (8), after incubation (9), after incubation + octopine (10).

2. *Petunia* protoplasts incubated with TI plasmids - Growth of cells from both plasmid-treated and control preparations declined on medium with reduced hormones. Subsequently, some colonies from plasmid-treated protoplasts (approx 1 in every 10^5 protoplasts originally plated after uptake) continued to proliferate on hormone-free medium. They appeared pale green against a background of brown, dying cells. The pale colonies were sub-cultured and have been maintained for over 2 yr on hormone-free medium with regular transfer every 4 wk. Control cells did not continue to grow after transfer to hormone-free medium, indicating that habituation is negligible in *Petunia*. Hormone-independent tissues selected from plasmid-treated protoplasts became intensely green, and in morphology and growth resembled crown gall tissues from authentic axenic tumours induced on *Petunia* stem explants by *ACH-5* bacteria. In comparison, non-transformed *Petunia* callus was creamy-white on medium with 2.0 mg/l NAA and 0.5 mg/l BAP. Tissues from plasmid-treated protoplasts grew without restraint when grafted onto the host plant. Such growths could be excised from the graft, returned to agar medium, and maintained in the absence of hormones. This property typifies crown galls (16) including authentic axenic *Petunia* tumours. Non-transformed *Petunia* callus failed to grow when grafted. Octopine and lysopine dehydrogenase were present in extracts of tissues from plasmid-treated protoplasts , the electropherograms being similar to those shown in Figs. 2 and 3, but not in extracts

of non-transformed *Petunia* callus.

DISCUSSION.

Transformation of higher plant protoplasts by isolated TI plasmids and of cells regenerated from protoplasts by intact *Agrobacteria* occurs at frequencies, higher when intact bacteria are used, which permit transformants to be selected by conventional plating techniques using hormone-free media. The advantage of using protoplasts as an experimental system is that it facilitates recovery of tissues of single-cell origin, this being reflected in the enhanced octopine content of transformed *Nicotiana* tissues compared to that of some uncloned authentic *Nicotiana* crown gall tumours. Since habituated tissues resemble crown gall tumours in their hormone independence, they are also recovered in the procedure used to select transformed cells. Habituation in control cultures must therefore be determined for any particular experiment. Comparison of the results reported indicates that habituation occurs at higher frequency in *Nicotiana* than in *Petunia*.

Intact bacteria have been used to transform tobacco suspension cells (17), and cells regenerated from leaf protoplasts of streptomycin resistant tobacco plants (18). The ability to transform cells regenerated from wild-type *Nicotiana* leaf protoplasts with intact *Agrobacteria* indicates that this transformation procedure is of general applicability to wild-type as well as mutant cell lines. The demonstration that isolated *Agrobacterium* plasmids can transform *Petunia* protoplasts (10), indicates that intact bacteria are not a prerequisite for tumour induction in culture. In view of this, it is now possible to use protoplasts and isolated TI plasmids to attempt to overcome the natural barrier to infection by *Agrobacterium* present in monocotyledons, and also to investigate the uptake of TI plasmids into protoplasts of fungi, bryophytes, and ferns. *Agrobacterium* plasmids may be suitable vectors with which to modify plant cells by the introduction of isolated genes (19, 20). Transposon Tn7 has been introduced into the T-DNA of the TI plasmid and detected in tobacco crown gall (21). It now remains to be seen whether such transposons will be expressed in transformed protoplasts and in regenerated plants.

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PROTOPLASTS AS A HOST CELL SYSTEM FOR PLANT VIRUSES

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A decade has elapsed since protoplasts of higher plants were first introduced into plant virus research as an *in vitro* system of host cells (1,2). By virtue of their facility to be infected synchronously and at high frequencies, protoplasts are now one of the standard experimental materials in a number of plant virology laboratories. It seems to be appropriate to review, at this stage, recent developments with this system to define its properties and to discuss some of the problems which have emerged from the findings using protoplasts. In this article, reference will be confined to the protoplasts from the mesophyll tissues of higher plants. There are several earlier review papers which are pertinent to the subject (3,4,5).

Recent Developments

The list of protoplast systems established for the study of plant viruses is growing steadily, and up to now protoplasts from at least 11 species have been successfully inoculated with more than 17 different viruses. Table 1 lists the plant viruses representing major taxonomical groups for which a system of synchronous infection has been made available using protoplasts. Important new additions to the list are tobacco necrotic dwarf virus (18) and cauliflower mosaic virus (19, 20). The former is closely related to potato leaf roll virus, and is, therefore, transmissible only with the aid of aphid vectors. Furthermore, it is confined to the phloem tissues in infected plants. The protoplast system established should undoubtedly be invaluable for studying the replication of this virus, a task extremely difficult to undertake with leaf material. It could be suggested that protoplast systems may also be developed for other viruses the inoculation of which is known to rely upon insects or other vectors. Cauliflower mosaic virus is unique in that it has a genome of double-stranded DNA. The high levels of infection recently attained with protoplasts (75-80 % of protoplasts infected (20)) and the high rate of replication (formation of inclusion bodies within 72 hrs (20)) should obviously be a great help for biochemical studies on the replication of the DNA genome.

TABLE 1 Plant viruses for which a synchronous infection system has been established using leaf protoplasts

Virus	Source of protoplasts	Reference
Tobacco mosaic	Tobacco	1
	Tomato	6
Tobacco rattle	Tobacco	7
Potato X	Tobacco	8
Turnip yellow mosaic	Chinese cabbage	9
Cowpea mosaic	Cowpea	10
	Tobacco	11
Cucumber mosaic	Tobacco	12
Brome mosaic	Tobacco	13
	Barley	14
	Radish	15
Alfalfa mosaic	Cowpea	16
Raspberry ringspot	Tobacco	17
Tobacco necrotic dwarf	Tobacco	18
Pea enation mosaic	Tobacco	19
Cauliflower mosaic	Turnip	20, 21

Protoplasts have also been inoculated with viroids, an infectious RNA of low molecular weight (23).

The inoculation method originally developed for tobacco protoplasts and tobacco mosaic virus (TVM) (1) has been found to be effective for many other combinations of viruses and protoplasts (5). Infection of protoplasts usually requires the addition of macromolecular polycations such as poly-L-ornithine. Exceptions to this general rule, however, have been found. Protoplasts can be infected in the absence of added polycations either when the viruses have higher isoelectric points and are therefore, positively charged under the inoculation conditions (pea enation mosaic virus (19) and brome mosaic virus (13,14,15)), or when the protoplasts have a relatively low negative surface charge (cowpea protoplasts (22)). These observations indicate that the initial adsorption of virus particles onto the surfaces of protoplasts depends on electrostatic forces, and support the idea that the primary function of polycations is to neutralize or

to reverse the negative charge of virus particles (5). It has recently been demonstrated that under inoculation conditions the virus particles form aggregates which presumably contain polycation and buffer molecules (24). These aggregates were, however, infectious to protoplasts only upon the addition of extra polycation, suggesting that the polycations have an additional function, possibly mediated by their binding to the surface of the protoplast (24). The effect of the inoculation buffer on the efficiency of infection of protoplasts has been studied to some extent. Phosphate buffer often gives rise to infection at higher efficiencies than citrate buffer (5, 26), but has the disadvantage that the efficiency tends to be affected by protoplast density (5, 25).

The mode of entry of virus into protoplasts has been a subject of controversy. On the basis of electron microscopic observations of thin sections, some workers proposed an endocytic process for virus entry (2,27), whereas others believed that virus enters protoplasts through polycation-induced lesions in the plasmalemma (28,29). The latter mechanism, however, does not explain infection by these viruses which have no polycation requirement and structures indicative of endocytic entry of brome mosaic virus has recently been observed in barley protoplasts inoculated in the absence of poly-L-ornithine (30). Infection in this system was enhanced considerably when the osmotic pressure of the medium was raised immediately before inoculation (31). This observation is also in line with the endocytic mechanism of virus entry, because the osmotic shock should reduce the turgor which counteracts the invagination of plasmalemma. The operation of an endocytic mechanism in isolated plant protoplasts was clearly demonstrated by the recent electronmicroscopic study of the uptake of polystyrene spheres in which the uptake was also shown to depend on an energy supply (32). It should be pointed out, however, that we still lack direct proof that the virus particles taken up by endocytic process are those which actually participate in the infection of protoplasts.

A remarkable improvement has been achieved with respect to the efficiency of infection of protoplasts by isolated viral nucleic acid. 30-40 % of tobacco protoplasts were infected with TMV-RNA at a concentration of 0.6 µg/ml (33), more than one thousand-fold improvement over the first report of infection of protoplasts with TMV-RNA (34). Brome mosaic virus RNA, at a concentration of 1 µg/ml, could give rise to infection of 90 % of barley protoplasts (30). The high efficiency in this case is due partly to the isolation and inoculation of protoplasts under slightly hypertonic conditions. Whether such conditions favour infection by RNA in other combinations of viruses and protoplasts remains to be seen. The improved efficiency of infection by RNA should permit the undertaking of more precise experiments than have previously been feasible on the role of individual RNA species of the viruses with divided genomes.

Finally there has been some progress in the method of isolation of protoplasts. The enzymatic isolation of mesophyll protoplasts usually requires manual removal of the epidermis before the leaves are subjected to enzyme treatment. We found that the

mesophyll tissues of tobacco can be dissociated simply by vacuum-infiltrating unpeeled leaf pieces with macerozyme solution followed by shaking at 25°C (I. Takebe and K. Shimidzu, unpublished). The resulting mesophyll cells (a mixture of spongy and pallisade cells) are subsequently converted into protoplasts by cellulase treatment. A preparation of macerating enzyme from *Aspergillus japonicus* appears to be a valuable addition to the list of enzymes used for protoplast isolation. The preparation (called Pectolyase Y-23) is unique in that it contains pectin lyase in addition to polygalacturonase. This enzyme can be used in both the two- and one-step procedures, and was found to be as active as Macerozyme R-10 at a concentration lower by an order of magnitude (35). Possible unfavorable effects of contaminating substances are minimal with Pectolyase Y-23 because of the low concentration required.

Basic Properties of Protoplasts as a Host Cell System for Plant Viruses

Being naked single cells suspended in a liquid medium, protoplasts have a number of unique properties which are not shared by conventional tissue materials. The most basic of them will be briefly referred to here, and their consequence in studies on plant viruses discussed.

Of greatest importance to plant virus studies is obviously the capacity of protoplasts to permit simultaneous infection of the majority of cells. In many instances around 90 % or more of the protoplasts are infected by brief contact with the virus, and, since there is little chance of secondary infection, the subsequent process of virus replication should proceed synchronously. It is this property that permits to the following of the course of infection events in individual infected cells, a task practically impossible with conventional tissue material. Such a study was made first using TMV in tobacco protoplasts (36,37) and it was shown that the synthesis of viral RNA starts within 4 hrs after infection. Viral RNA accumulates in the early phase of infection as free or partially assembled RNA. In the later phases, when the synthesis of coat protein becomes intensive, however, viral RNA is assembled into virions as soon as it is made. In addition to coat protein, two large polypeptides (140,000 and 180,000 d) are synthesized in infected protoplasts. These polypeptides have sizes similar to the products of *in vitro* translation of TMV-RNA, and are therefore most probably virus-coded. Coat protein is made in much higher amounts than the large polypeptides, and its synthesis becomes active later than that of the latter. These findings clearly indicate that the mode of *in vivo* translation of TMV-RNA is not of the monocistronic type known for picorna viruses of animals, in which the entire span of viral RNA is translated into a very long polypeptide (polyprotein) which subsequently undergoes specific proteolytic cleavage to produce individual gene products.

Similar studies were more recently made with tobacco rattle virus (38), cucumber mosaic virus (39), cowpea chlorotic mottle

virus (40) and brome mosaic virus (41) in tobacco protoplasts, and with cowpea mosaic virus in cowpea protoplasts (42). Some of these studies represent the first documentation of how RNA species of the viruses with divided genomes are synthesized in infected cells. Whereas, coat protein is the only viral protein detectable in infected plant materials, several virus-specific proteins have been found in the protoplast systems and their course of synthesis studied. With potato virus X in tobacco protoplasts, the formation of characteristic inclusion body was shown not to precede the appearance of progeny virus (43), thus eliminating the possibility that the structure is the site of synthesis of virus components.

Because protoplasts are single plant cells completely separated from each other, they represent a system which is strictly at the cellular level. This is another basic property of protoplast systems, and its significance is particularly clear when one studies the interaction between two viruses. Infection of plants by more than one virus is quite common in nature, but, except in a few cases, it is not clear whether two different viruses invade the same cells or they infect different cells in the same plant. In contrast, the immunofluorescence technique applied to protoplasts has clearly demonstrated that two unrelated viruses can infect the same cell, and that there is little if any interference between them in the infection of protoplasts (44). Furthermore, tobacco protoplasts were shown to be infected at high frequencies even by two closely related strains of TMV (45). This finding clearly invalidated the"exclusion hypothesis" which claimed on the basis of experiments with leaf materials that only one TMV particle can participate in the infection of a tobacco cell. Progeny virus particles produced in the doubly infected protoplasts have been examined for antigenic constitution by electron microscopic serology, and it was found that they are coated with a mixture of proteins of the two strains (45). Protoplasts previously infected by one strain of TMV, but not those infected by an unrelated virus, were found to be refractory to subsequent infection by another strain of TMV (46). This observation seems to provide a basis on which the mechanism of interference between related viruses is to be studied. A similar finding has been reported more recently with two strains of raspberry ringspot virus (47).

Being a system at the cellular level, protoplasts are also useful for disclosing aspects of infection specifically associated with tissues. Thus, one of the genes conferring resistance to TMV infection to tomato plants was found not to be expressed phenotypically in tomato protoplasts; protoplasts carrying this gene were as susceptible to infection by TMV as the protoplasts from susceptible lines of tomato (48). It is highly probable that the function of this gene is to interfere with an infection process at a tissue level, possibly the cell-to-cell movement of virus. Most of the cowpea varieties immune to cowpea mosaic virus were likewise susceptible to infection, when they were tested as protoplasts (49). A recently isolated temperature-sensitive mutant of TMV multiplied freely in protoplasts even at non-permissible temperatures at which it barely multiplied in leaves. Examination by immunofluorescence technique

revealed that in the inoculated leaves kept at non-permissible temperatures virus multiplication was confined to primary-infected cells, showing that the mutation is in the viral gene controlling the spread of virus in leaf tissues (50).

In tobacco leaves fully infected by TMV, most of the leaf cells are saturated with progeny virus, but there are small areas, called green islands, where little virus is present. When protoplasts were isolated from such leaves, a renewed virus multiplication occurred in the cells which already contained enormous amounts of virus, and vigorous virus replication also took place in the protoplasts from green islands (51). This observation seems to suggest that some unknown mechanisms operate at a tissue level to restrict the multiplication of virus.

Problems Emerged from the Findings with Protoplasts

The basic assumption underlying the use of protoplasts in plant virology is the belief that the process of virus replication, and associated events, are basically the same as those in the cells of intact leaves. While the assumption seems to be largely correct, instances have been found where infected protoplasts behave differently from the cells in leaves. A "classical" example is the lack of necrotic response to TMV infection in tobacco protoplasts (52). The leaves of tobacco varieties carrying the N gene form local lesions upon TMV infection as a result of necrotic collapse of infected cells. Protoplasts of the same varieties, however, supported as active virus multiplication, and remained viable as long, as control protoplasts lacking the N gene. This unexpected observation was interpreted as suggesting that a certain type of cell-cell interaction is involved in the expression of the N gene (52), and this idea was supported by the recent report that necrotic lesions are not formed even in the leaves, if cell-to-cell contact, possibly through the plasmodesmata, is broken by plasmolyzing the tissues (53). However, nothing is known about the postulated cell-cell interaction, and other explanations do not seem to have been excluded.

It should be noted in this respect that evidence is accumulating to show that the metabolism of isolated protoplasts differs in some aspects from that of leaf cells *in situ*. A remarkable rise in RNase level due to *de novo* synthesis was demonstrated in tobacco protoplasts (54), whereas the synthesis of fraction I protein was found to decrease markedly upon isolation of protoplasts (55). Isolation of protoplasts is also accompanied by changes in the level and the pattern of polyribosomes (56). Some of these changes were ascribed to hyperosmolarity of the medium in which protoplasts have to be maintained (54), while others are probably the result of interruption of the nutrient supply (56). The metabolism of protoplasts may also be affected by other factors such as the absence of cell walls (57) and the trauma caused by treatment with crude enzymes. It is clear that further studies are needed to characterize the metabolism of protoplasts. Some of the peculiar

behavior of protoplasts with regard to virus infection might turn out to be the result of a specific metabolic pattern characteristic of isolated protoplasts.

Protoplasts thus far inoculated successfully with plant viruses derived from those tissues and species which had been known to be invaded by the viruses. However, instances have recently been reported which deviate from this. As already mentioned, tobacco necrotic dwarf virus is confined in plants to the phloem tissues, none of the mesophyll cells being infected. In contrast, mesophyll protoplasts of tobacco are readily infected by the same virus and support vigorous virus multiplication (18). This finding clearly indicates that the absence of virus in the mesophyll tissues is not due to an inherent inability of mesophyll cells to support virus multiplication. Interesting questions therefore arise as to whether some sort of barrier exists which prevents the invasion of mesophyll cells in leaves, or alternatively, an unknown mechanism in leaves makes the mesophyll cells immune to this virus.

Most of the hosts of brome mosaic virus are within the Graminae, and only a few species outside this family are infected by this virus. It has recently been reported, however, that protoplasts of radish leaves are readily infected by brome mosaic virus to produce significant amounts of virus (15). Parallel trials to inoculate radish leaves with the same virus were unsuccessful; infection of leaf cells could be detected neither by biological assay nor by isolating protoplasts from the inoculated leaves followed by examination using the immunofluorescence technique. This finding calls for a need to reexamine the concept of host range of plant viruses, and at the same time points to the possibility of using protoplasts of "non-host" plants as a host cell system of plant viruses.

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LEGUME ROOT NODULE PROTOPLASTS

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ABSTRACT

With an enzyme mixture comprising pectate lyase (EC 4.2.2.3), a hemi-cellulase, and a cellulase (EC 3.2.1.4), bacteroid containing protoplasts can be isolated from the root nodules of Centrosema pubescens, Glycine max, Pisum sativum, and Vigna unguiculata. The technique is also suitable for the production of protoplasts from young roots. A nodule protoplast system from V.unguiculata was selected for further study. It possessed a functional tricarboxylic acid cycle, a glycolytic pathway, and was capable of asparagine and glutamine inhibitable acetylene reduction. Culture on a variety of different media, with or without hormones, failed to induce cell-wall or callus formation, nor did the protoplasts synthesize DNA. Minor amounts of RNA and protein were elaborated however, and an occassional bacteroid completed division.

Theoretically, root nodule protoplasts should form the simplest symbiotic system between legumes and Rhizobium. Davey et al.(1) first described their isolation from nodules of Glycine max, while Hoh and Broughton (2) modified their technique for use with other plants. Both these groups failed to demonstrate an active nitrogenase enzyme (EC 1.7.99.2) within the protoplasts. Later, Broughton, Wooi, and Hoh (3) demonstrated acetylene reduction (a measure of nitrogen fixation) in root nodule protoplasts of V.unguiculata after a lag period of 24 to 48 h. As far as could be judged, the acetylene was reduced by bacteroids within the protoplasts. The purpose of this communication is to report further properties of this system especially with regard to the relationship between carbohydrate metabolism and nitrogen fixation, and factors which control nitrogen fixation.

MATERIALS AND METHODS

Protoplasts were isolated from root nodules (inoculated with UMKL 76) as follows (see ref 4):
Freshly picked nodules were washed extensively with sterile distilled water, sliced tangentially, and immediately immersed in the following: "Rhozyme HP 150" (a hemi-cellulase), 1 % (w/v); pectate lyase (EC 4.2.2.3), 0.5 % (w/v) in basal medium. Composition of the basal medium was: sorbitol, 0.6 M; HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid), 0.05 M; potassium dextran sulphate, 0.3 % (w/v); KNO_3, 1 mM; KH_2PO_4, 0.2 mM; $MgSO_4$, 0.1 mM; $CaCl_2$, 0.1 mM; KJ, 1.0 µM; $CuSO_4$, 0.1 µM (pH 5.8).

In all incubations, the ratio of enzyme solution to nodule slices was about 10 to 12 ml per gram. After vacuum infiltration, the slices were incubated on a horizontal rotary shaker (100 rev.min^{-1}) at 25°C for 1 h. Then the supernatant was discarded, the tissue slices washed in sterile basal medium, and teased apart. The outer ring of cortical tissue surrounding each slice was discarded, and the bacteroid containing cells were incubated in basal medium containing 0.5 % (w/v) pectate lyase and 1 % (w/v) bovine serum albumin for a further 5 h. The mixture was then filtered through fine stainless-steel gauze (about 100 µM mesh), and the protoplasts released in 1 % (w/v) cellulase (EC 3.2.1.4) dissolved in basal medium (1 h incubation). Protoplasts were allowed to settle under gravity, and washed free of contaminating rhizobia by serial decantation (about twelve times) in twenty volumes of sterile basal medium without KNO_3 (3). Actual handling of the protoplasts was restricted to a minimum, and made as gentle as possible. If the protoplasts were required for later use, they were stored in basal medium and washed extensively just before use. Contamination was checked routinely by plating protoplast samples on the agar medium of Child and LaRue (5) which also contained 0.6 M sorbitol.

Acetylene reduction was assayed as described by Williams and Broughton (6), respiration was followed by measuring carbon dioxide production with a gas chromatograph (4), and the various metabolic activities expressed on a dry weight of protoplast basis (4). Various substances (carbohydrates, hexose phosphates, pyruvate and tricarboxylic acid cycle intermediates, amino acids and nucleotide phosphates) were added to the protoplast suspensions as described by Wooi and Broughton (4). Nucleic acid and protein metabolism was followed by adding ^{14}C-thymidine, ^{3}H-uridine and ^{14}C-valine to protoplast suspensions, incubating for various periods of time, collecting the cells on glass-fibre discs, washing the discs free of unreacted label with trichloroacetic acid and ethanol, and counting them in a scintillation solvent (6). Protoplasts were cultured on a variety of media based on that of Murashige and Skoog (7), but also containing sorbitol (0.5 M), sucrose (2.5 % w/v), casein hydrolysate (0.1 % w/v). Other compounds (2.4-dichlorophenoxyacetic acid - 0 to 5 ppm), benzyl adenyl purine (0 to 1.0 ppm), naphthylene acetic acid (0 to 5 ppm) and thiamine (1 ppm) were also added in many different concentrations, and combinations.

RESULTS

Root-nodule protoplasts of V.unguiculata are heterogenous in both size and shape (Fig.1). They range in length from 35 to 135 µm and in width from 35 to 95 µm. About 5 x 10^{6} protoplasts settle into a volume of 1 ml. Yields were in the range of 30 to 50 mg dry weight of protoplasts per gram fresh weight of nodules. The absence of a cell wall was shown by fluorescence microscopy, and the ability to burst under pressure (4).

Routine checks showed little or no bacterial contamination when protoplasts were plated on agar suitable for the growth of G.max callus. We take this, together with the facts that: (a) a lag period in acetylene reduction is always observed in freshly isolated, but not older, protoplasts, and (b) we have never observed ^{14}C-thymidine incorporation into protoplasts (suggesting no bacterial multiplication), to mean that the acetylene reduction observed (Fig.2) occurs within the protoplast. Whilst these criteria effectively rule out nitrogen fixation associated with bacterial contaminants, we cannot exclude the possibility that the acetylene reduction observed occurs in bacteroids imbedded in the cytoplasmic matrix. Under the microscope, a

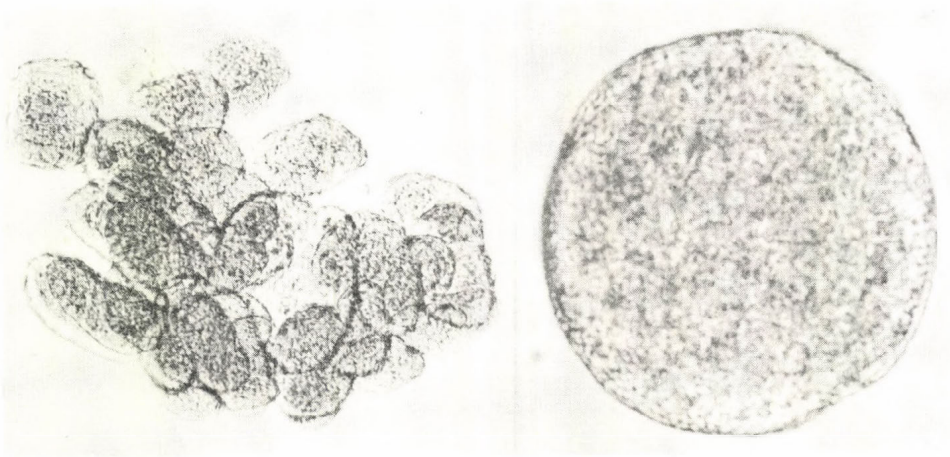

Fig. 1. a. Light micrograph of a group of freshly isolated V.unguiculata protoplasts. b. Micrograph of a single isolated protoplast. The granular appearance is due to bacteroids.

large proportion of cells appear intact, but we can see no way of showing that these, and these alone are the ones responsible for the acetylene reduction.

In basal media, acetylene reduction develops 24 to 36 h after protoplast

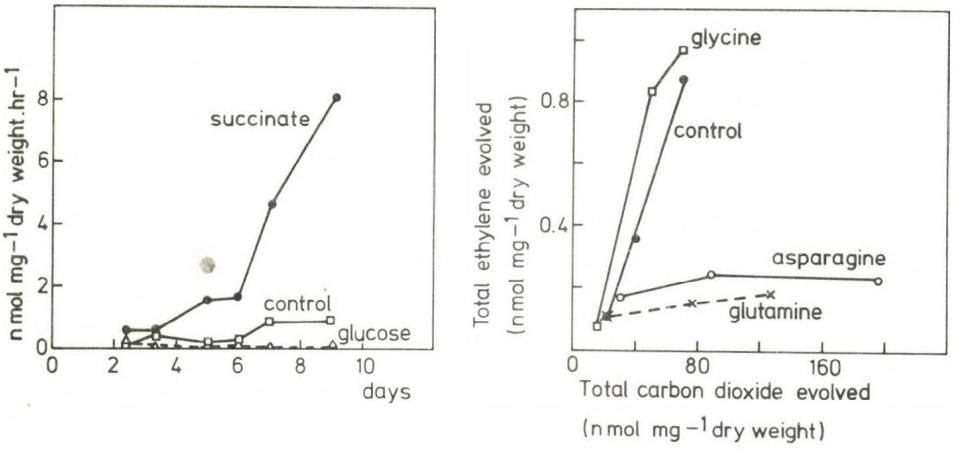

Fig. 2.a. Effect of glucose and succinate (40 µmol to 1.5 ml of gravity packed protoplasts) on the development of acetylene reduction activity in V.unguiculata root nodule protoplasts. b. Effect of various amino acids (final concentration 1.08 x 10^{-2} M) on the rate of acetylene reduction of V.unguiculata root nodule protoplasts. Protoplasts were 2.5 d old at the beginning of the experiment. In both cases "Control" samples were incubated in basal medium, and each point represents the mean of duplicate determinations.

isolation, and continues to increase in activity over a period of several days (Fig.2a). Various additions to the basal medium have varying effects on the rate of both acetylene reduction and respiration. Of the various carbohydrates tested, arabinose, glucose and myo-inositol had little effect and in some cases glucose was inhibitory. This was not true of the phosphorylated glucose derivatives, or of fructose-1,6-diphosphate which markedly stimulated acetylene reduction but had little effect on carbon dioxide production. Similar effects were observed with citrate, malate, and phospho-(enol)pyruvate. Amino acids had effects ranging from markedly stimulatory (lysine) to inhibitory (asparagine and glutamine). Again, these effects were less marked with respect to respiration, which both asparagine and glutamine stimulated. Apart from UTP, all the nucleotide triphosphates stimulated both acetylene reduction and respiration, though some like ATP enhanced acetylene reduction more than carbon dioxide production. Other adenine phosphates like AMP, cAMP, ADP etc., stimulated respiration more than acetylene reduction. Acetyl phosphate had no effect on acetylene reduction (the phosphoroclastic reaction is absent from symbiotic nitrogen fixing systems).

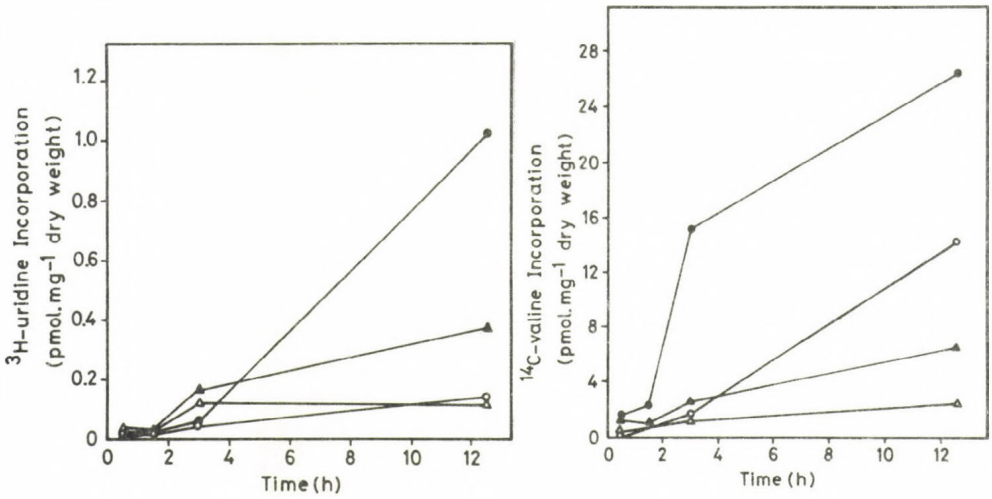

Fig. 3. a. Rate of incorporation of ^{3}H-uridine into RNA by protoplasts incubated in Murashige and Skoog (7) medium. b. Incorporation of ^{14}C-valine into trichloroacetic acid insoluble products by protoplasts incubated in Murashige and Skoog medium (7). Ages of the protoplasts were as follows: ●1 d; ○3 d; ▲5 d; and △7 d old. Results are means of duplicate determinations.

The rate of L-valine incorporation into TCA-precipitable products by protoplasts incubated in Murashige and Skoog medium (with 0.5 M sorbitol) decreased with increasing age of the samples (Fig. 3b). No appreciable L-valine incorporation was observed in protoplasts incubated in basal medium. Taken together these data suggest that neither medium is suitable for the prolonged growth of the protoplasts. Protoplast samples incubated in Murashige and Skoog medium (with 0.5 M sorbitol) incorporated minor amounts of uridine into RNA (Fig. 3a). The rate of RNA synthesis reached a maximum in three day old protoplasts and declined thereafter. Culture of protoplasts of

different ages on a variety of media failed to induce cell wall formation or callus growth. There was no evidence for DNA synthesis in all the protoplast samples tested.

DISCUSSION

A heterogenous population of bacteroid-containing protoplasts can be isolated from the root nodules of V.unguiculata.

Preliminary experiments have shown that the technique is also suitable for their isolation from the root nodules of Centrosema pubescens, Glycine max, and Pisum sativum. With strict control of the procedure, axenic cultures can be produced in about 12 h. Under appropriate conditions, tissue imbedded in the cytoplasmic matrix of these protoplasts will reduce acetylene to ethylene. Intermediates of the glycolytic pathway and the tri-carboxylic cycle stimulate acetylene reduction, while asparagine and glutamine inhibit it. Intra-experimental variability, as measured by acetylene reduction, is at the most twenty to twenty-five percent, and often less. Such variation is within the limits of that normally observed with Rhizobium infected callus cultures (cf 8).

At first sight, the declining RNA and protein synthesizing capability suggests that the protoplasts may be moribund. In vivo protein synthesis declines with increasing age of bacteroids however (9). Senescence may therefore be a property of aging bacteroids than of the system per se. Lack of DNA synthesis, as well as declining RNA and protein synthesis undoubtedly account for the failure of the protoplasts to regenerate cell walls and to divide in culture.

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THE BIOPHYSICS OF PROTOPLAST FUSION

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Abstract

Protoplast fusion results from the coalescence of cell membranes and the mixing of cytoplasms following contact. Zeta potentials at the protoplast surface govern approach, while interfacial energies regulate the consequences of contact. The interfacial energies of protoplasts from Zea mays and Aureobasidium pullulans have been measured by a new technique involving polymeric aqueous coacervate systems. Spreading tensions correctly predicted the type of fusions which occurred. There was a direct relation between the expected free energy of fusion, calculated from surface energies, and the observed free energy of fusion, calculated from equilibrium coefficients. These results demonstrate the utility of surface physics in the prediction of the degree of success to be expected from cell fusion media.

I. Introduction

The fusion of protoplasts is one of the most promising and widely applicable methods of genetic manipulation known today. Successful protoplast fusion experiments have yielded new strains of Saccharomyces and have resulted in inter-specific crosses such as the tomato-potato hybrid reported by Melchers et al. (5).

The methodology of fusion, however, is poorly defined and unpredictable at this time. Usually, protoplasts are fused in relatively high concentrations of polyethyleneglycol (e.g. 30%). It is the purpose of this paper to discuss the biophysical principles of protoplast fusion and to demonstrate how these physical principles apply to the formation of interspecific fusions between protoplasts from Zea mays (monocot) and Aureobasidium pullulans (fungi).

II. Theory

Interspecific protoplast fusion requires the approach, contact and coalescence of 2 different types of protoplasts. These are physical events rather than biochemical ones, and

the appropriate description of this process is given in terms of surface physics. In this section we will describe the predominant physical events occurring during fusion.

Approach between protoplasts is primarily determined by the long-range electrostatic forces resulting from the zeta potential present at the cell surface. The achievement of intimate contact occurs when the van der Waals forces acting at the protoplast surfaces begin to overlap. The net van der Waals forces determine the interfacial energy at the protoplast-medium interface. The result of this interaction can be either complete or partial protoplast fusion, or the total failure of attachment and coalescence. This second step is thus determined by the interfacial energies of the protoplasts.

It is important to recognize that these considerations are thermodynamic in nature and thus describe the final, equilibrium condition with disregard for kinetics.

1. Zeta potentials:

The zeta potential is the apparent net surface charge of a particle suspended in a fluid. It is determined from the electrophoretic mobility of the particle. Zeta potentials of similar particles from a single source tend to be similar in both sign and magnitude for a given suspension medium, although for biological cells there is always a distribution of zeta potentials. For instance, the zeta potential of red blood cells in physiological saline is -18 mV (8).

Particles having high zeta potentials of the same charge will not approach closer than the point at which the forces of Brownian motion equal those of electrostatic repulsion. In practice, it is found that contact between particles does not occur unless average zeta potentials are reduced to 0 ± 5mV. Reduction of the zeta potential to within this range allows contact and flocculation in such diverse system as clay suspensions, dye suspensions, the products of biological waste treatment, bacterial suspensions, and cell suspensions.

Methods of reducing zeta potentials include pH adjustment, the well-known Schultz-Hardy rules (e.g. the use of di- and trivalent ions), and the use of polyionic polymers which both neutralize the zeta potential and bridge between the suspended particles. Addition of both charged and neutral polymers often aids the kinetics of aggregation, but prevents intimate contact between the particles. The importance of pH and Ca^{++} (most cells have a negative zeta potential) in protoplast fusion (11) supports consideration of cell aggregation in terms of flocculation. Polyethyleneglycol may enhance cell aggregation by bridging, but also certainly affects the interfacial tension between the cells and the surrounding medium (see below).

2. Surface energies:

Once intimate contact between protoplasts has been established, the final equilibrium outcome is entirely depend-

ent on the interfacial energies (γ_i) between the protoplasts and the medium. Measurement of the interfacial energy at the cell surface will be discussed below. For the fusion of dissimilar protoplasts in a given fusion medium, the three interfacial energies which totally determine the outcome are: γ_{P_1M}, γ_{P_2M} and $\gamma_{P_1P_2}$, where for example γ_{P_1M} is the interfacial energy between protoplast 1 (P_1) and the medium (M).

The consequences of a given set of interfacial energies can best be determined from the spreading energies (S), which are calculated with equations 1a,b,c.

$$S_1 = \gamma_{P_2M} - (\gamma_{P_1M} + \gamma_{P_1P_2}) \qquad (1a)$$

$$S_2 = \gamma_{P_1M} - (\gamma_{P_2M} + \gamma_{P_1P_2}) \qquad (1b)$$

$$S_3 = \gamma_{P_1P_2} - (\gamma_{P_1M} + \gamma_{P_2M}) \qquad (1c)$$

where S = spreading energy

P_1 = protoplast type 1

P_2 = protoplast type 2

M = medium

γ = interfacial energy.

In each case, the spreading energy gives the tendency for a given phase to spread on a second phase while both are immersed in a third. If the spreading energy is positive, spreading will occur, otherwise it will not occur (9).

In any given system, at least one spreading energy will be negative, it is a useful convention to designate this spreading tension as S_1. The three possible combinations of spreading energies given in Table 1 correspond to the 3 possible results of the interaction of 2 different protoplasts in a liquid medium (Fig. 1). Combination (a) results in the complete failure of fusion.

Table 1: Effect of Spreading Energies on Protoplast Fusion

	Spreading Energies			
	S_1	S_2	S_3	Consequence for Fusion
a)	<0	>0	>0	Complete Separation
b)	<0	<0	<0	Partial fusion
c)	<0	<0	>0	Complete fusion

Table 1. Three combinations of spreading energies in a mixture of 2 types of protoplasts. By convention, $S_1 < 0$.

Combination (b) results in partial fusion or clumping. Combination (c) results in either complete engulfment of one protoplast type by the other or in complete fusion of the

cells. The fate of the engulfed protoplast is determined by other factors which are discussed below.

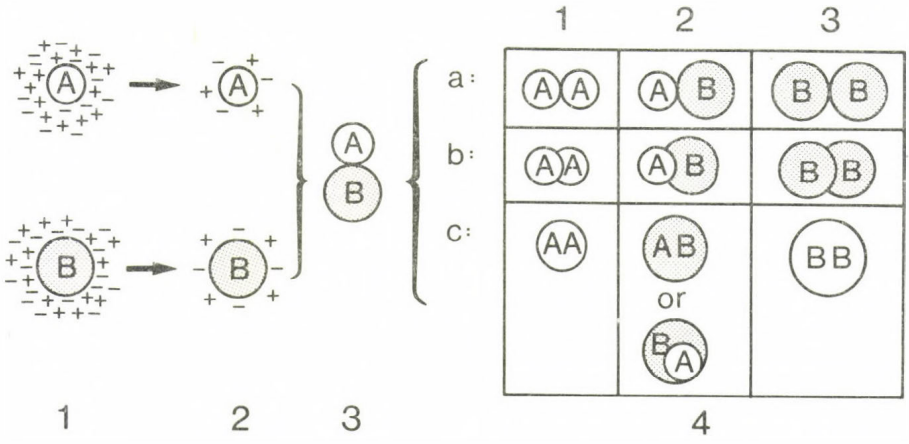


Figure 1. Pathways of protoplast fusion.

Figure 1 diagrams these possibilities and summarizes the processes leading to fusion. In step 1 to 2, zeta potential is reduced; in step 2 to 3, aggregation is promoted; and in step 3 to 4 the consequences of the surface energies take place. In stage 4, the coalescence of like protoplasts (1-c and 3-c) is much more likely then non-coalescence (1-a, 1-b, 3-a and 3-b), which generally results from a kinetic problem and will only result when the membranes are highly viscous. Likewise, coalescence of unlike protoplasts can result either in the complete mixing of the different cytoplasms or in engulfment without mixing (2-c). Engulfment without mixing can result from either a highly viscous membrane surrounding the engulfed protoplast, or coacervation phenomena (1). The interaction of unlike protoplasts given in Figure 1, step 4 corresponds directly to the 3 combinations of spreading energies given in Table 1.

The basis for the measurement of the surface energies of cells has been given by Gerson (2) and Van Oss, et al. (8), but will be summarized below.

III. Methods

Surface physics:

Surface tension (γ_{LV}) was measured with a de Nouy surface tensiometer having a strain guage transducer. Contact angles were measured directly from magnified images of the drop resting on a surface.

The method for determining the surface energy of protoplasts was as follows. Protoplasts were collected from an appropriate suspension medium on a Millipore filter which was removed from the filter holder before all the liquid had passed in order to prevent drying of the protoplast surface.

This was immersed in the suspension medium in a Costar 3025 culture flask from which the upper surface had been removed. Drops of either FC-40 (a fluorcarbon liquid of low surface tension, γ_{LV}=16 dynes/cm, produced by 3M) or a matching coacervate (see below) were placed on the layer of protoplasts and the contact angle was measured. Great care was taken to prevent drying of the protoplasts, as problems resulting from drying are a major experimental difficulty in the work of van Oss et al. (8).

Calculation of the surface energy of the protoplasts can be accomplished from contact angles in this 2-phase liquid-liquid system provided the interfacial tension between the liquids is known. Given these, the interfacial energy between the protoplasts and each phase can be calculated using the method of Neumann, et al. (6). This method has been shown to have a high degree of consistency and utility in both biological and engineering applications.

The use of coacervates to measure the interfacial tension of cells has not been reported previously. In order to determine the surface energy of a plane surface, it is necessary to be able to form a drop having a finite contact angle on that surface. This occurs when the interfacial energy between the surface and the surrounding medium is less than that between the drop and the surrounding medium. In the case of cells in an aqueous medium, the interfacial energy is often very low (e.g. 1.0-0.01 ergs/cm^2). This requires that the interfacial energy between the 2 immiscible liquids be similarly low. The pure liquids with the lowest known surface energies are fluorcarbons (e.g.FC-40), they are also of relatively high density which facilitates measurement. However, the interfacial tension is not low enough, and contact angles on cells are often very high (e.g. 150^{o}). Greatest accuracy in this method is obtained for contact angles between about 45^{o} and 135^{o}.

To solve the problem, it was necessary to find pairs of immiscible, non-toxic, relatively osmotically inactive liquids. One approach is to use fluorcarbons or hydrocarbons with surfactants, but often these will cause cell lysis. An entirely satisfactory and highly versatile solution to the problem is to use polymer systems demonstrating coacervation, or phase separation. Mixtures of this type are in wide use for cell and particle separation (1), and, in fact, separate cells and particles according to their surface energy (10).

Here, a mixture of 5% (w/v) polyethyleneglycol (PEG; 20,000 MW) and 5% Dextran (Pharmacia; 500,000 MW) was used to form the 2-phase system. The upper phase consisted primarily of dextran, while the lower phase was primarily PEG. Protoplasts were immersed in the light phase and drops of the heavy phase were placed on the protoplast layer; contact angles were well within the limits required for accurate determinations of surface energy. A great advantage to the use of 2-phase polymer systems is that the interfacial tension between the phases is adjustable by altering the concentrations of the

compounds (1). Thus, the optimal contact angle range for a given cell surface can usually be obtained without having to resort to a totally different 2-phase system. In addition, it is advantageous to use aqueous solutions for the determination of cell surface energy (10).

Measurement of the interfacial energy between the coacervate phases is potentially difficult. Albertsson(1) used the spinning drop technique (7), which is generally difficult and at best problematical in the case of 2-phase polymer systems because of centrifugal effects. A useful and new method for measuring low interfacial tensions between liquids is to measure the contact angle of a drop of one liquid immersed in the other on a surface of known surface energy. It is then relatively simple to calculate the interfacial tension between the liquids using Neumann's equations (6). Again, to obtain accurate results, an appropriate solid must be chosen so that contact angles are between 40° and 135°. For aqueous polymer systems, agar and other gels give good results, but the solid of choice in this case was Nylon 6-6. Using this method, the interfacial energy between the 2 phases of the 5% PEG - 5% dextran system used here was found to be 1.01 ergs/cm^2.

Protoplasts and fusion:

Protoplasts from Zea mays were prepared according to the method of Meadows and Walden (3,4). Briefly, this method is as follows. Seneca 60 seedings were grown for 21 days in the greenhouse, and placed in a dark, humid chamber 16 hours prior to use. The unrolled leaves surrounding the apex were floated on a solution of 0.5% cellulase (Onozuka R-10) in 9% mannitol and Zapata's salts at pH 5.5. This was shaken at 125 RPM for 3 h at 23°C, filtered and centrifuged. Protoplasts were washed twice in medium lacking cellulase. Protoplasts from Aureobasidium pullulans were prepared with 0.5% Driselase under anaerobic conditions from non-pigmented yeast-like cells in 20% sucrose, 50mM Tris and 10mM $MgSO_4$ at pH 7.3. Fusion was performed in this medium plus 30% (w/v) polyethylene glycol (20,000 MW).

IV. Results

Surface energies of protoplasts:

Using the 2-phase polymer system, the contact angles of the dense phase on the protoplast surface were measured. For Zea mays protoplasts the contact angle was 66.4° and for protoplasts from A. pullulans, the contact angle was 130.7°. From these data alone, it is relatively safe to conclude that Ap protoplasts will be engulfed by Zm protoplasts in PEG, however a full analysis is as follows.

Table 2 gives the interfacial energies of both types of protoplasts in the PEG-rich and dextran-rich phases. In the PEG-rich phase, the interfacial energy of Zm protoplasts is 0.092 ergs/cm^2, and that of Ap protoplasts is 0.691 ergs/cm^2. With respect to this phase, Ap protoplasts are much more hydrophobic than Zm protoplasts.

Table 2: Surface and Interfacial Energies of Protoplasts

surface properties in PEG/dextran*	Protoplast type Zm	Ap
contact angle	66.4°	130.7°
γ_{P1}	0.092	0.69
γ_{P2}	0.500	0.031
$\gamma_{P\text{-air}}$	55.44	55.47
$\gamma_{P_1P_2}$	0.28	

* 1=PEG-rich phase, 2=dextran-rich phase, γ=ergs/cm^2

The type of interaction to be expected in the PEG-rich phase can be determined from the spreading energies, as indicated above. Spreading energies are given in Table 3 for each phase using the standard convention. For the PEG-rich phase, one would predict complete engulfment of Ap protoplasts by Zm protoplasts, for the dextran-rich phase the opposite may occur but is geometrically hindered.

Table 3: Spreading Energies of Protoplasts in PEG/Dextran

Spreading energy (ergs/cm^2)	Phase System PEG (A)*	Dextran (B)*
S_1	- 0.88	- 0.75
S_2	- 0.50	- 0.25
S_3	+ 0.32	+ 0.19

* A. $\gamma_{P_{Ap}L_1} > \gamma_{P_{Zm}L_1}$: 1 = Ap, 2 = PEG, 3 = Zm

* B. $\gamma_{P_{Zm}L_2} > \gamma_{P_{Ap}L_2}$: 1 = Zm, 2 = Dextran, 3 = Ap

The free energy of engulfment can be calculated from these data as well and gives an indication of the yield of fusion products to be expected (assuming reversibility and equilibrium). For the engulfment of Ap protoplasts by those of Zea mays, the net free energy change is given by equation (2).

$$\Delta G_{net} = \gamma_{P_{Zm}P_{Ap}} - \gamma_{P_{Ap}L_1} \simeq -RT\ln \frac{[Zm/Ap]}{[Zm][Ap]} \qquad (2)$$

Disequilibrium or the presence of undetermined effects (e.g. surface charge or surface roughness) may add another free energy term to the right-hand side of equation 2, thus, exact equality probably will not be observed, but proportionally between the free energy calculated from surface energies and that calculated from concentrations should be observed.

b) Fusions:

Fusions were performed as described above in PEG and the

engulfment of Ap protoplasts by Zm protoplasts was observed (Fig. 2).

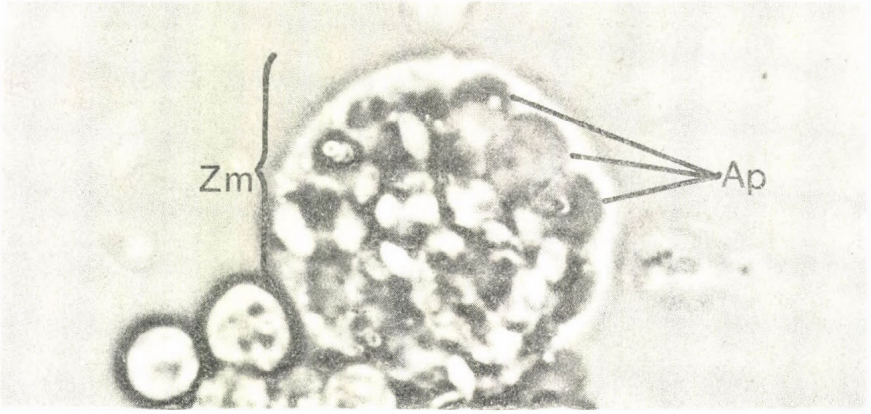


Figure 2. Engulfment of protoplasts of Aureobasidium pullulans (Ap) by a protoplast of Zea mays (Zm).

Counts of the numbers of the various combinations are given in Table 4.

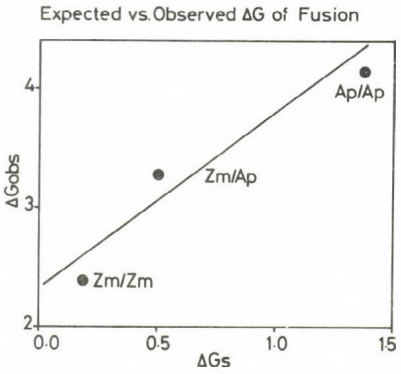
Table 4: Fusions of Zm and Ap Protoplasts

Combination	% observed
Ap/Ap	62%
Ap/Zm	27%
Zm/Zm	11%

Intraspecific fusions between Zm protoplasts were less common from either interspecific fusions or Ap intraspecific fusions. This result is related to the surface energies of the protoplasts. Figure 3 gives the relation between the free energy of adhesion between the 3 combinations (ΔG_s), calculated from only the interfacial energies, and the observed free energies of fusion (ΔG_{obs}) determined from equation 2. As expected, there is a linear relation between the observed fusions and that predicted from the interfacial energies. The results in Figure 3 demonstrate that consideration of interfacial energies allows qualitative prediction of the outcome of a cell fusion experiment. The slope and intercept of the line in Figure 3 depend on specific characteristics of both the cells and fusion conditions.

Engulfment of Ap protoplasts by Zm protoplasts did not result in the dissolution of the Ap protoplast in the Zm cytoplasm. This could result either from a high interfacial energy at the Zm cytoplasm - Ap membrane interface, a high Ap membrane viscosity or coacervation phenomena involving the constituents of the 2 cytoplasms.

Figure 3. Relation between observed and expected free energies of fusion.



V. Conclusions.

It has been demonstrated that both intraspecific and interspecific fusions of protoplasts from Zea mays and Aureobasidium pullulans are predictable from the interfacial energies of these protoplasts in the fusion medium. A new method for determining the interfacial energy at the cell surface has been described involving aqueous polymeric coacervates such as polyethyleneglycol and dextran. In addition, a new method has been described for determining the interfacial energy between immiscible liquids by the use of a reference solid. Clearly, the application of surface physics to the methodology of cell fusion could allow rapid screening for the determination of the optimal composition of fusion media.

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PROTOPLAST AS A USEFUL TOOL FOR STUDYING PLANT PLASMA MEMBRANE MICROVISCOSITY

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INTRODUCTION

The plasma membrane is the major barrier separating the cells from their environment and its importance in the regulation of a number of processes at the cellular level has long been recognized. The lipid constituents of the bilayer and their physico-chemical states are believed to be involved in the control of membrane function. In contrast to animal cells, our information about the composition and transitional state of the outer plasma membrane of plant cells (the plasmalemma), largely due to technical difficulties encountered during its isolation, are fragmentary and indirect (1). Since the plasmalemma has functions rather different from those of the endoplasmic reticulum or chloroplasts, efforts to learn more about its constitution and physico-chemical state are warranted. A unique possibility to gain information about the behaviour of the plasmalemma during phase transition is to incorporate spin labeled fatty acids in the plasma membrane of isolated protoplasts and to study the motional relations in the membrane under various physiological conditions.

MATERIALS AND METHODS

Plants. Leaves of 7-day-old *Triticum aestivum* L. cv. Miranovskaya 808 and cv. Penjamo 62 were used for protoplast preparation as described earlier (2). The leaves cut into 0.5 mm pieces were incubated with 0.5 M glucose and 1.4 mM $CaCl_2$ at pH 6.0 for 3x20 minutes. Then the cells were digested with 2 % Onozuka R-10, 0.5 % Rhozyme and 1 % pectinase in a buffer solution containing 3 mM MES, 0.6 mM NaH_2PO_4, 7 mM $CaCl_2$, and 0.5 M sorbitol at pH 6.4 (3). After a suitable incubation time the protoplasts formed were sedimented by centrifugation (1000 rpm), washed, and resuspended in the above buffer. The average yield was around $5x10^6$ protoplasts per g of leaf tissue.

Spin labeling technique. The stock solution (in chloroform) of spin labeled fatty acid (2-/14-Carboxytetradecyl/-2-ethyl-4,4-

-dimethyl-3-oxazolidinyloxyl, a Syva product) was evaporated onto the bottom of an Erlenmeyer flask, and shaken with the above buffer containing 5 % bovine serum albumin, in a volume to give 0.1 % solution of fatty acid. Usually 16 hours were necessary to complex all the fatty acids (4). 7.0×10^6 protoplasts were gently shaken in the presence of 200 µl buffer solution containing the spin label enriched BSA for one hour. Then the unreacted spin label was removed by repeated washings and gentle centrifugations. Finally, the protoplasts were taken up in 100 µl of the original buffer. The viability of the protoplast suspension was not affected by the spin label treatment.

The ESR spectra were taken with a JEOL JES-PE-1X spectrometer. Evaluation of the spectra was according to (5). Results are expressed in terms of rotational correlation time, τ_C, which is directly proportional to the microviscosity of the system. Higher values represent more rigid membranes.

RESULTS AND DISCUSSION

The protoplast suspension, after proper incubation in spin label containing buffer, exhibited an ESR signal characteristic of ordered lipid layers (Fig. 1a). As far as the localization of

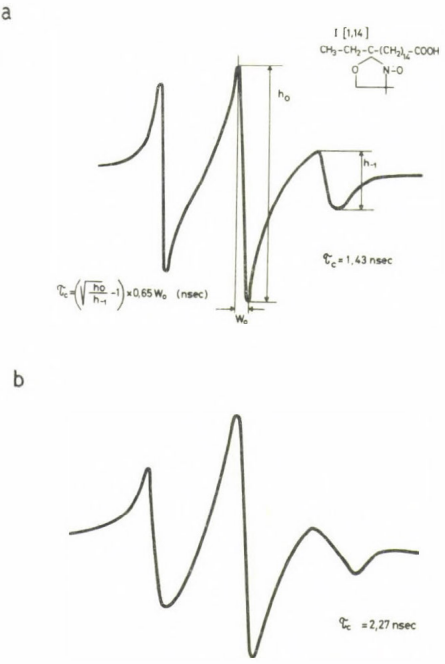

Figure 1. Typical ESR spectrum of the protoplast (a) and chloroplast (b) suspension

In the upper right hand corner the structure of the spin probe used is given.

the spin probes is concerned, the most likely candidates are the plasma membrane and the chloroplast membrane which amount to 10 % and 65 %, respectively, (6) of the total cell membrane surfaces. It is very important to note that because intact cells were investigated the outermost plasma membrane was the main target, and there was only a minor contribution from the chloroplasts since the nitroxide group was readily reduced in the cytoplasm. This qualitative observation was confirmed by the comparison of the ESR spectrum of the protoplasts (Fig. 1a) and that of chloroplasts isolated from the same protoplast suspension (Fig. 1b); the observed spectra could be best fitted with significantly different spectrum parameters (e.g. order parameter and rotational correlation time). On the basis of these findings we conclude that the observed spectra can be ascribed exclusively to spin probes embedded in the plasmalemma.

Indirect evidence in favour of the above conclusion was provided by experiments in which the effect of Ca^{++} on membrane fluidity was measured. Calcium, similarly to other bivalent cations, is known to bind in the lipid head-group region, thereby condensing the interface, which results in an increase in membrane microviscosity (7). Since the membrane is impermeable to excess calcium, Ca^{++} affects primarily the outermost membrane, the plasmalemma only. As shown in Fig. 2, the motional freedom of the spin probes gradually decreased with increasing calcium concentration. The incubation medium used for protoplast isolation also contained Ca^{++}. Thus, it is interesting to speculate how, by manipulating the physico-chemical state of the plasmalemma, the relationship between the transitional state and the membrane functions, particularly the role of Ca^{++} in protoplast fusion (8), could be studied.

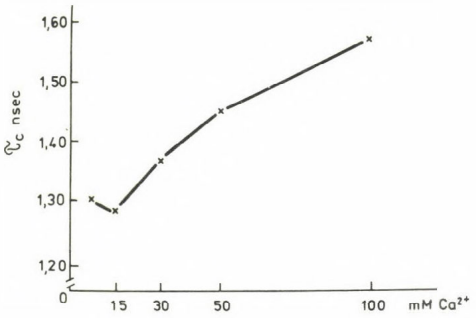

Fig. 2. The effect of Ca^{++} on the fluidity of the plasma membrane of isolated protoplasts of Miranovskaya 808.

To characterize the transitional state of the plasmalemma the ESR spectra were recorded between 5 and 32°C in 1°C intervals. As shown in Fig. 3 there is a break in the Arrhenius plot of the correlation time at 18-20°C indicating a thermotropic phase transition.

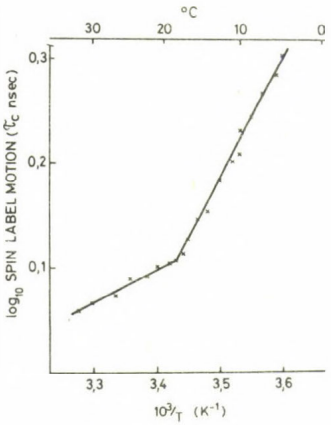

Fig. 3. Arrhenius plot of the correlation time in isolated protoplasts of Miranovskaya 808.

The lipid composition and, thus, the reported phase transition temperature of different membrane structures of the plant cell varies considerably. Using spin label method, Raison et al. (9) have found that the thermotropic phase transition temperature is 10-12°C in mitochondria; Murata et al. (10) with the aid of a fluorescent technique, have found the transition temperature to be 30°C, in chloroplast suspensions.

When comparing the results, and considering our observation on the marked difference between the fuildity of the plasmalemma and the chloroplast membrane, we cannot but conclude that the physico-chemical state of the different cell membrane structures should be entirely different, depending on their functions, even within the same plant cell. It has to be added that whenever the spin probes are introduced from one (the outer) side only, taking the slow flip-flop rates into account (11), we mainly monitor the outer monolayer of the plasmalemma which is probably different from the inner one due to the asymmetric distribution of the constituting lipids. However, the physical state of membranes may vary in different physiological conditions.

Senescence has previously been reported to bring about a decrease in the fluidity of prokaryotic (12), mammalian (13), and plant (14) membranes. As shown in Fig. 4, the correlation time, as measured in the plasmalemma of protoplasts from both the first and the second leaves of wheat, exhibited a gradual change during ageing. The decreasing trend in fluidity (e.g. increasing "correlation time") was very much the same in different cultivars (Miranovskaya 808 and Penjamo 62) when compar-

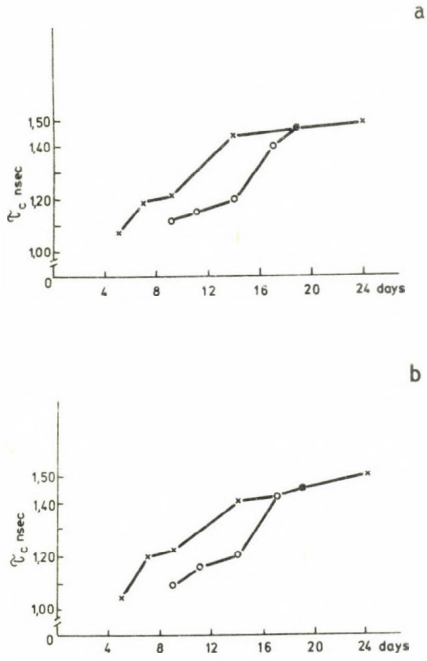

Fig. 4. Changes in the membrane fluidity during senescence.
a Miranovskaya 808,
b Penjamo 62,
x First leaf,
o Second leaf.

ing the same leaves at similar ages. It should be noted that the second leaf completely repeats the fluidity-time profile of the first leaf. Whether these correlation time data are indicative of shifts or broadenings in the phase transition remains to be seen.

CONCLUSIONS

In situ spin labeling of the plasmalemma of isolated protoplasts provides information on the transitional state of these membrane structures. We demonstrated that the observed ESR signal is exclusively due to spin probes embedded in the plasmalemma, and so this method overcomes the difficulties encountered during the isolation of plasma membrane fragments from plants. However, the conventional method of isolating plasma membranes for chemical analysis is still necessary. One of the most promising aspects of our method is a new possibility to study the effect of membrane modifying substances on the transitional state of these structures. This method should effectively contribute to the clarification of many problems and is most valuable where signals due to intracellular structure cause serious interference (e.g. Fluorescence studies, (15)).

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LABELLING AND ISOLATION OF PLASMA MEMBRANE FROM HIGHER PLANT PROTOPLASTS

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INTRODUCTION

Protoplasts are potentially an ideal starting material for the isolation and characterization of plant plasma membranes. The absence of the cell wall obviates high shear force disruption, while allowing the use of non-penetrating surface labelling methods analogous to those applied to animal systems. However, the absence of morphological markers and the equivocal nature of enzymic markers for higher plant plasma membrane emphasises the need for reliable and specific methods of identifying this membrane in sub-cellular fractions.

Differential and sucrose gradient centrifugation methods have been widely used in higher plant plasma membrane isolation studies with intact tissues as source material(1). Phosphotungstic acid/chromic acid staining (PTAC)has been widely used as a marker for plasma membrane(1,2,14)and has been positively correlated with certain enzymes, notably K^+-ATP-ase and glucan synthetase. However, this stain(and STAC,(3))has been shown to be unreliable in some cases(4,5,6) when used for tissue or protoplast staining, while the validity of K^+-ATP-ase and glucan synthetase as useful markers has also been disputed (7,8).

Conventional separation methods have been used in this study employing several subcellular enzymic markers and in addition, the non-penetrating cell surface markers concanavalin A(conA) and lanthanum (La).

MATERIALS AND METHODS

Surface labelling:
Leaf protoplasts of Nicotiana tabacum var.Xanthi were isolated (9) and used in 0.6M sorbitol, 1mM $CaCl_2$ and sometimes surface-labelled with ^3H-conA (10) or 2.6% w/v $LaCl_3$(9). After protoplast fractionation, ^3H was counted by scintillation while La was assayed by neutron activation analysis. Samples containing La were neutron irradiated at 2 x 10^{13} neutrons sec^{-1}. cm^{-2} for 2h and counted against standards at 1.76 MeV.

Fractionation:
For differential centrifugation, protoplasts were broken in 30ml 0.6M sorbitol by hand-shaking for 1 min and sequentially centrifuged at 3000 x g (3KG) x 5 min,13000 x g(3-13KG) x 10 min, and 193000 x g(13-193KG) x 30 min. Pellets were resuspended in 1mM TES-Tris,pH 7.0.For discontinuous sucrose gradient centrifugation, protoplasts were broken in 1ml 0.6M sucrose using 3 strokes of a needle and syringe. Broken protoplasts or differential cen-

trifugation fractions were loaded onto gradients of composition:25,27,29,31 33,35,40% w/w sucrose in 75mM MES-Tris,pH 7.5. The gradients were centrifuged at 97000 x g x 130 min. Gradient fractions were offloaded, diluted x ½ with MES-Tris and recentrifuged at 193KG x 30 min. Pellets were resuspended in MES-Tris.

Biochemical assays:
All enzyme assays were incubated at 25°C.

Chlorophyll: 80% acetone extracts were read at 652nm(11).
Protein: NaOH solubilised samples were estimated against BSA fraction V standards (12).
Cytochrome c oxidase: by the method of Smith (13).
NADH cyt c oxidoreductase: by the method of Hodges and Leonard (1).
Glucan synthetase: High (230µM,no $MgCl_2$) and low (6µm, 10mM $MgCl_2$). UDPG substrate concentrations were used, with UDP ^{14}C-glucose as the tracer. NaOH soluble and insoluble extract radioactivity was counted.
ATPase: 2mM Na_2ATP± 2mM $MgCl_2$ and 50mM KCl were used, while 3mM pNPP replaced ATP as a control.
IDPase: Fractions without storage were assayed at pH 7.5 with 2mM IDP, 1mM $MgCl_2$.

Electron Microscopy:
Embedded protoplasts and fractions were examined by transmission e.m.(6).

RESULTS

The activity and distribution of various enzymes, protein and chlorophyll in differential centrifugation fractions appear in Table 1.

TABLE 1. Distribution of enzymes and markers after differential centrifugation

Pellet fraction	Protein	Chl.	Cyt c.ox.	Glucan synthetase substrate conc. Low		High		IDPase	ATPase basal	+Mg^{++}	+$Mg^{++}K^{+}$	ConA	La
3KG	94	98.5	44	54	27	49	12	45	62	47	39	63	75
3-13KG	4	1.2	47	22	37	28	49	29	20	23	26	19	11
13-193KG	2	0.3	9	24	36	23	37	26	18	30	35	18	14
				NaOH insol		NaOH Insol.							

All quantities are percentage of total particulate activity recovered in the fractions

The distribution of protein and chlorophyll indicated that the large organelles(e.g.chloroplasts)sedimented at low R.C.F.while that of cytochrome c oxidase indicated that the majority of mitochondria were pelleted by an R.C.F.of 13KG. Electron microscopy confirmed this and additionally indicated that nuclei and Golgi bodies were largely confined to the 3KG pellet.The 13-193KG pellet was free of recognisable organelles except for ribosomes; the low levels of chlorophyll and cytochrome c oxidase activity in this fraction confirmed this and indicated that the fractionation procedure had resulted in minimal organelle disruption. Although most markers(except cytochrome c oxidase)occurred in the 3KG pellet, substantial enrichment on a protein basis was present in the less dense 3-13KG and 13-193KG pellets, largely because chloroplast Fraction 1 protein, which sedimented in the low R.C.F.fractions, heavily contributes to the calculation.

Using discontinuous gradients, broken protoplasts were separated into 7 fractions and the distribution of enzymes and substances assayed is shown in Fig.1. Clumping of the subcellular material did not occur although the majority of the material appeared in the denser fractions. Protein and chlorophyll distribution was similar to that for conA, cytochrome c oxidase,

NADH cytochrome c oxidoreductase and high substrate glucan synthetase. All forms of ATPase were distributed more towards the lighter fractions, while low substrate glucan synthetase appeared mainly in the lighter fractions with a second peak in denser fractions. When protoplasts were surface labelled with La, and then centrifuged, the distribution of protein (similar to that of La) was shifted to denser fractions.

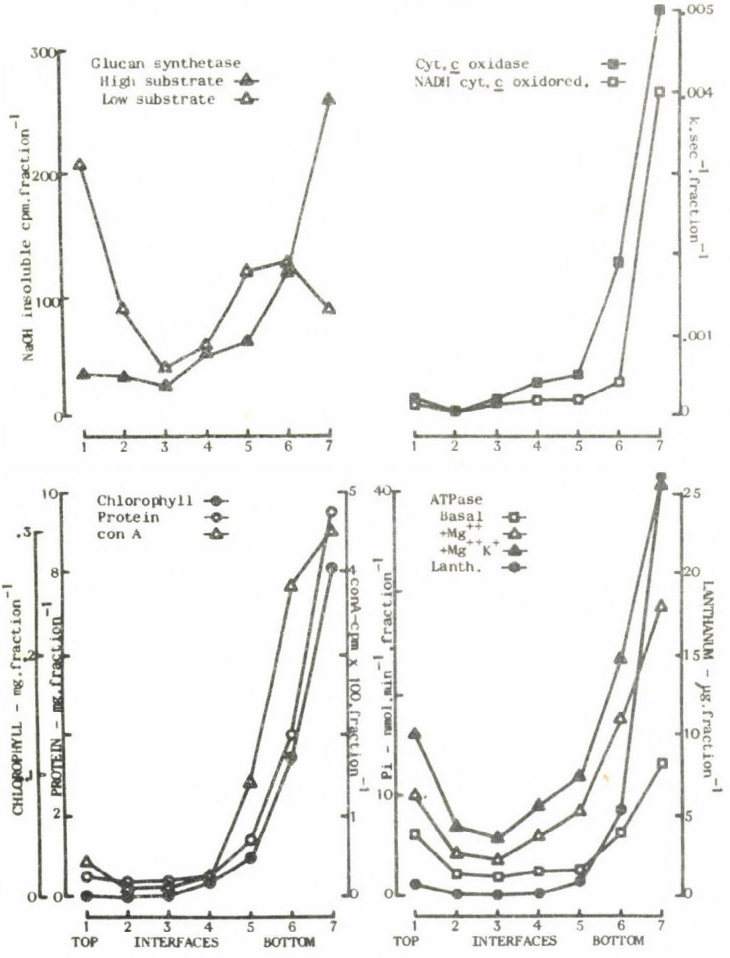

Figure 1: Distribution of broken protoplasts loaded on a sucrose gradient: Activity of enzymes and content of substances recovered from interface fractions. Interface : 1 2 3 4 5 6 7

Fractions (% w/w sucrose) overlay, 25, 27, 29, 31, 33, 35, 40

The use of discontinuous gradients was taken further by loading resuspended 3-193KG or 13-193KG pellets from protoplasts previously labelled with La. Again, no clumping was seen, although the distribution of protein (Fig.2), indicated that the "microsomal" fraction (13-193KG) did not sediment as far in the absence of the 3-13KG fraction. The La distribution was significantly different, displaying two peaks in lighter and denser fractions in the "microsomal" gradient separation. The distribution of a control non-La labelled "microsomal" fraction down a gradient was very similar to that of a

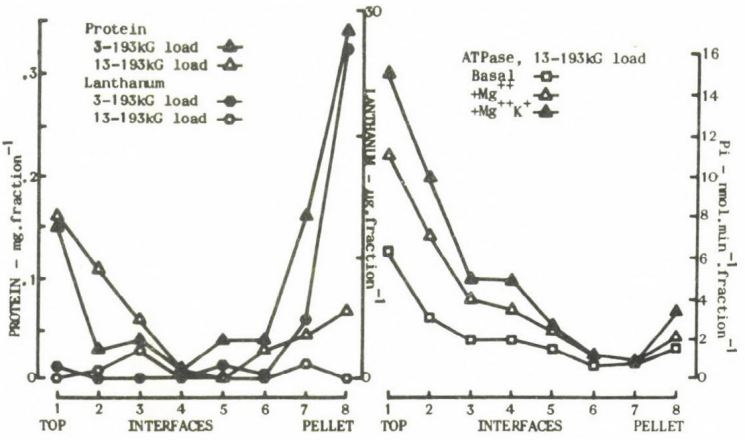

Figure 2: Distribution of 3-193KG and 13-193KG pellets loaded on a sucrose gradient:Protein,ATPase and La content of fractions.

La labelled fraction. ATPase activity(Basal,Mg^{++},$Mg^{++}+K^{+}$) of fractions from a "microsomal" loaded gradient did not indicate a qualitative enrichment of any form of ATPase in any fraction(Fig.2).pNPPase distribution was similar to that of ATPase. The pH curve of ATPase for a "microsomal" fraction was similar in many respects to that of an intact vacuole preparation, prepared from similar protoplasts.

Electron microscopy of La labelled protoplasts indicated that the stain did not penetrate intact protoplasts (Fig.3), or transfer onto organelles. Only otherwise featureless membrane was found to be stained with La in subcellular fractions (fig.4).

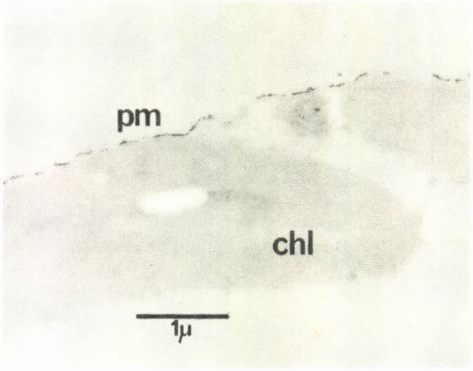

Figure 3:Part of a protoplast labelled with La.No post stain.

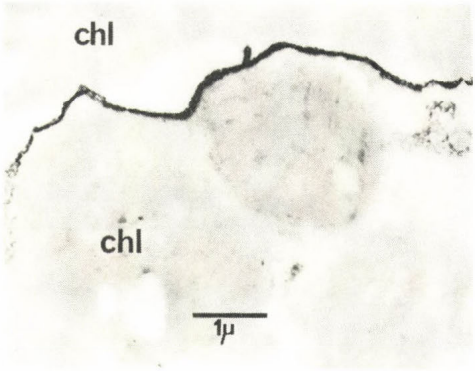

Figure 4: A 3KG pellet from protoplasts previously labelled with La. No post stain.

DISCUSSION

The methods currently in use for plasma membrane isolation from plants have been developed on the assumption that K^{+}-ATPase is an integral component of

this membrane and that it is unequivocally stained by PTAC. A good correlation between K^+-ATPase enrichment and PTAC(and glucan synthetase) has been the basis for claiming that a particular subcellular fraction is enriched in plasma membrane (2,14) although this has recently been questioned(4,5,6). Methods using low shear forces have undoubtedly been developed more slowly than they might have been owing to the acceptance of plasma membrane isolation methods using an intact tissue source based on PTAC criteria (14). Recently, protoplasts have been increasingly used as a source of organelles (15,16,17,18), including plasma membrane(19) using the surface label diazotised sulphanilic acid. No independent proof that this label was irreversibly bound to plasma membrane was presented in this study; however this method of labelling holds promise.

The conventional sucrose gradient centrifugation of a "microsomal"fraction does not yield plasma membrane free from contamination from other subcellular sources. When employed in the fractionation of protoplasts where shear forces were minimised, these methods still do not allow the preparation of a "pure" plasma membrane fraction. $LaCl_2$ has been shown to be a satisfactory electron dense plasma membrane stain which does not appear to transfer to other subcellular loci during cell disruption (6,9). La can be assayed quantitatively and is thus potentially a very useful marker both in cytochemical and biochemical studies. ConA is also a non-penetrating label, particularly useful when conjugated with gold(10),but so far has not been investigated as a plasma membrane marker in subcellular fractions in higher plant work. Using La as a tentative reference marker, the results of the differential centrifugation indicated that plasma membrane was present in all fractions recovered and although most was found in the 3KG pellet, this fraction is unattractive as a plasma membrane source because of substantial contamination with all other subcellular structures. The most attractive fraction was the 13-193KG "microsomal" pellet containing 14% of the La and 18% of the conA label. This, however, also contained substantial contamination by mitochondrial debris and probably endomembranes.

Loading entire broken protoplasts onto gradients ensured that no fraction has been prematurely discarded. The results indicated that the size range of plasma membrane fragments was considerable and that the larger fragments were trapped between organelles during centrifugation. The distribution of plasma membrane down the gradient closely matched that of chlorophyll and protein, hence a useful enrichment of plasma membranes from this type of separation system is unlikely. There was evidence that Mg^{++}- and $Mg^{++}K^+$-ATPase was enriched in less dense fractions and thus not directly correlated with La, but high substrate glucan synthetase was found to be correlated with La and perhaps strengthens the case for this activity as a possible plasma membrane marker (8). No separation of the 13-193KG fraction into different ATPase species was observed, indicating that a range of membrane fragment size and density possessed this enzyme. The distribution of La in such gradients showed two peaks, further suggesting a lack of correlation of plasma membrane with ATPase. The difficulties of separation of plasma membrane from other membranes in a homogenate may not directly depend upon the shear forces used, as there is probably a more or less constant relation between the size of membrane fragments and organelle fragments over a large range of shear forces. Entrapment may thus be a constantly present problem.

Progress in the field of plasma membrane isolation will depend upon the development of surface affinity methods of isolation which include poly-L-lysine coated beads (20) and cyanogen bromide activation of polysaccharides (preliminary results of this laboratory). This approach will be greatly

helped by the concurrent use of reliable surface labels for plasma membrane such as La,conA and diazotised ^{35}S-sulphanylic acid. Protoplasts will, of course, be the essential starting material for these procedures.

ACKNOWLEDGEMENT

We thank the Agricultural Research Council for financial support.

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MICROTUBULES IN HIGHER PLANT CELLS AND PROTOPLASTS

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INTRODUCTION

An understanding of the processes of cellular differentiation is essential for any rational manipulation of plant development in tissue cultures. Some such processes which can be identified are the control of cell division versus control of differentiation which are often suggested to be mutually-exclusive events (1). We have chosen to study this balance in a simple tissue culture system in which rapidly dividing cells do not elongate whereas under different conditions elongating cells do not divide. This balance can be manipulated by altering the level of 2.4-D and some conclusions are already emerging on the structural basis of cell elongation. In this study, protoplasts and partially degraded cells have been used as material for vizualising by immunofluorescence microtubules which support elongated cell shape or form the mitotic spindle. The long term aim of the work is to see how such elements are involved in controlling division as well as shape in tissues.

MATERIALS AND METHODS

Fine suspension cultures of Daucus carota L. were grown in Murashige & Skoog's medium (GIBCO) containing 1 p.p.m. 2.4-D, 3% sucrose; 5% coconut milk and 0.5 p.p.m. kinetin. Elongated cells were produced by: i) allowing the culture to enter stationary phase; ii) by diluting or removing 2.4-D from the medium; iii) by arresting the cells in G1 by the addition of FUdR which blocks DNA synthesis. Onozuka R-10 cellulase was purified on a Sephadex G-75 column (unpublished). Dissolved in growth medium plus 0.25M sorbitol, this enzyme was used for about 30 minutes to degrade partially the cell wall or was used for 60 min to make protoplasts. These were attached to polylysine-derivatized coverslips and extracted in a microtubule-stabilising buffer (100mM PIPES pH 6.9; 1mM $MgSO_4$; 2mM EGTA; 0.25M sorbitol) containing 1% Triton X-100. After 20 min they were fixed briefly in formaldehyde and excess aldehyde neutralized. Affinity-purified rabbit antibodies to bovine tubulin were then added and visualized with fluorescein-conjugated goat anti-rabbit serum. (For fuller details see 2.)

RESULTS AND DISCUSSION

DIVISION VS. ELONGATION AS A FUNCTION OF THE CELL CYCLE

Some as yet unpublished work provides an understanding of how elongated cells form. Rapidly dividing cultures contain small clusters of essentially isodiametric cells (although some elongated cells are always inherited by subculturing). Frequent sub-culture into medium containing 1 p.p.m. 2.4-D maintains this state but subculture into 0.01 p.p.m. 2.4-D does not sustain rapid division and instead the cells vacuolate and elongate. By diluting the 2.4-D in this way a range of cell lengths can be obtained which varies as an inverse function of 2.4-D concentration. Cell counts also show that cell division and cell elongation are inversely related. Other workers have suggested that plant cells may elongate in G1 (3,4) and we suggest that carrot cells do so when there is insufficient 2.4-D to effect the transition from G1 to the (S→M) block of events involved with cell division. In support of this, FUdR can be added to block the entry into Sphase (this can be reversed by thymidine) and cells can be shown to elongate at concentrations of 2.4-D that would otherwise produce rapidly dividing cultures of isodiametric cells. Such concentrations of 2.4-D do not, therefore, inhibit elongation directly. In addition, EM confirms that cytoplasmic microtubules are conspicuous in the isodiametric cells, suggesting that high 2.4-D does not prevent directional enlargement by a direct effect on the cytoskeleton. We therefore conclude that cell elongation is a process initiated in G1 (as a normal part of growth) and which is cut short when threshold levels of 2.4-D trigger the transition into those phases of the cell cycle associated with division.

MICROTUBULES AND DIRECTIONAL CELL ENLARGEMENT

Indirect immunofluorescence shows that carrot protoplasts contain a network of fibrils (Fig 1). The use of affinity purified anti-tubulin; abolition of staining by absorption of serum with tubulin; staining of the mitotic apparatus; location of colloidal gold-tagged antibodies along linear tubular structures in negatively stained preparations; sensitivity to colchicine, all confirm the microtubular nature of the 'fibrils' (2, and unpublished). Using only partly degraded elongated cells (instead of protoplasts) transverse, branched hoops of microtubules can be located (2) which better reflect the native arrangement of MTs seen by EM (Figs 1B,1C). In the electron microscope, microtubules are observed as groups of about four, immediately beneath the plasma membrane. For this reason, the 'branched' fibrillar hoops are more likely to be bundles of microtubles than individual tubules and this is demonstrated in Fig 1D. Bright spots are occasionally seen at the nodes of these branches which may be microtubule organising centres associated with microtubule polymerization (see Fig 1B).

These and other observations raise questions of how MTs control the shape of cells, and in this connection the study of protoplasts provides some interesting points. For instance, removal of the wall from elongated cells produces spherical protoplasts which contain MTs (Fig 1D). MTs by themselves do not therefore control cell shape (see also 5). Microtubules cannot be detected by immunofluorescence in cells treated for 24 h with 10^{-3} M colchicine even though they retain their elongated shape at that time - implying that it is the cell wall which maintains this shape.

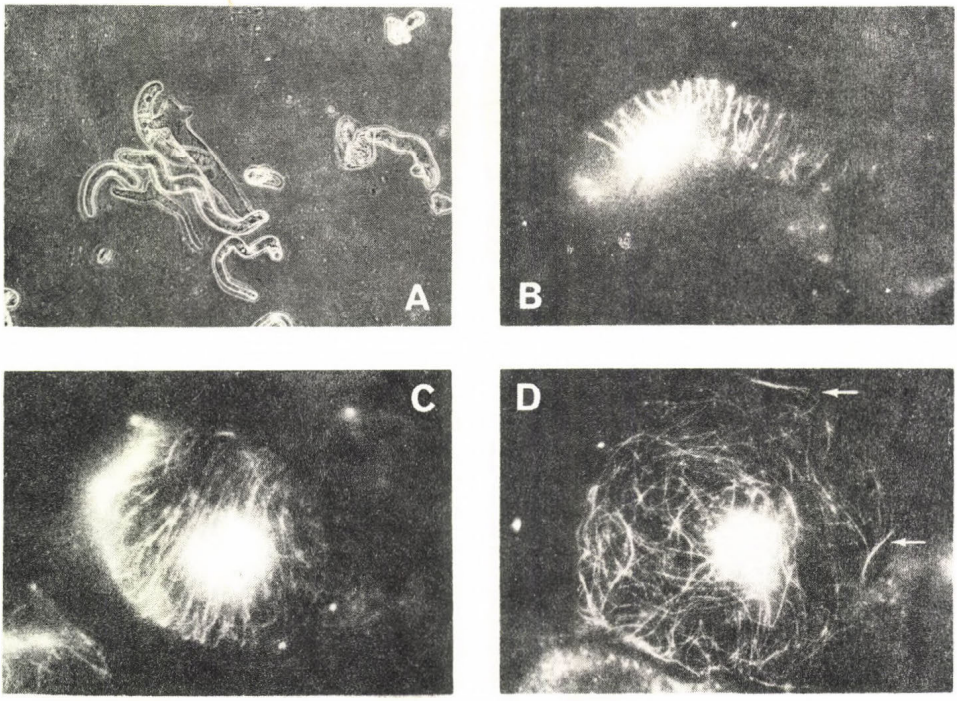

Figure 1 Immunofluorescence of elongated cells at different stages of cellulase treatment

1A. Elongated cells in culture; phase contrast x 100

1B. Elongated cell after minimal cellulase treatment to allow penetration by antibodies. Thick, anti-tubulin-staining 'fibrils' are seen in transverse, branched hoops. Note the bright areas at branch points; x 750 (Reproduced by kind permission of Nature).

1C. After further cellulase treatment, elongated cells begin to lose shape. Microtubules may still be seen in transverse array but the fibrils are thinner and not so tightly organized as in 1B; x 750

1D. When wall is completely removed to form a spherical protoplast, microtubules are no longer to be seen in ordered arrays. They remain, however, as a network in which few free ends are observed. Arrows denote where thick 'fibrils' can be seen to fray into several thinner components; x 750

It is concluded that microtubules overlap and associate side-to-side to form a continuous fibrillar network which, in the elongated cell, takes the form of several, thick, branched hoops. Some association with the plasma membrane and/or cell wall may be necessary for stabilising these hooped configurations (and vice versa).

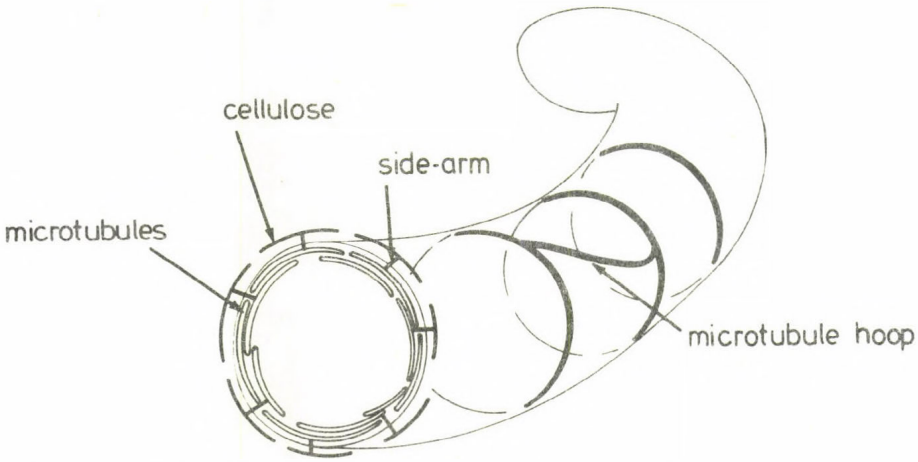

Figure 2 Schematic view of MT/wall interaction in controlling plant cell shape

Present observations are consistent with Heath's (6) model in which mobile, membrane-associated cellulose synthesising enzymes are directed through the membrane by microtubules at the cytoplasmic face of the plasma membrane. Cellulose biosynthesis is not dependent upon the presence of MTs but where the mobility of the enzyme is confined to the path provided by MTs, cellulose deposition reflects the orientation of MTs. Microtubule hoops may therefore be considered as a shape-determining template. Microtubule-microtubule side-bridges stabilise the hoops and MT-plasma membrane bridges (7,8) would connect the tubule to cellulose. To account for the MT re-orientation which we observe in the round to elongate transition, it is suggested that MTs slide along one another. Movement of hoop components relative to one another could, in this way, impart a slow pitch to the deposited cellulose.

However, after longer periods, the cells tend to become spherical. It is inferred that during active cell enlargement cellulose fibrils are only deposited in an orderly manner consistent with an elongated cell shape where they are under some influence provided by microtubular hoops.

It has often been noted that cytoplasmic microtubules parallel the arrangement of cellulose microfibrils in the wall and a direct transmembrane control of the direction of cellulose deposition by MTs has been proposed by Heath (6). His suggestion is that a transmembrane cellulose synthetase complex is associated with microtubules which provide the tracks along which mobile enzymes run, thereby depositing the linear cellulose microfibrils in a pattern dictated by the underlying MTs. We, and others (5), find that microtubules remain attached to sheets of membrane obtained from broken protoplasts. This implies some membrane/MT interaction (such MT side-arms have been seen in EM (7). We also find that microtubules remain upon the polylysine-treated coverslips even when the membrane sheets which bear them are delipidated by treatment with up to 10% Triton X-100. Such MTs seem to be stably attached to the glass and although direct evidence in favour of the postulated transmembrane assembly complex is lacking these observations hint at its possible existence.

But whatever the mechanism of microtubule/cellulose interaction, morphological evidence argues for a prime role of microtubule hoops in determining the elongated shape of plant cells and unpublished EM studies reveal that MTs are not random in 'round' cells but already exist as hoops before the cells elongate. Given that plant microtubules do overlap (i.e. do not appear to be anchored to a common organizer) and are seen to be bridged to one another and to the plasma membrane (7,8) by side-arms, circular bundles of MTs might simply be the natural consequence of MT polymerization and association in isodiametric cells. EM shows MT bundles are arranged either equatorially in 'round' cells or in two separate planes. Since MTs in elongated cells are predominantly transverse, re-alignment of some of the hoops evidently occurs during cell enlargement.

Microtubules in plant cells may control shape; be involved with the orientation of wall materials; are seen to anticipate the plane of division and may influence the deposition of the cell plate. Their involvement in all these processes suggests a function for them in controlling the morphology of cells and tissues. However, the polymerization and depolymerization of cytoplasmic MTs and mitotic spindle MTs is one characteristic of the cell division cycle. In yeasts, replication of a microtubule organising centre signals the entry of the cell into DNA replication (9) and there is other evidence to implicate MTs is growth regulation in other eukaryotic cells (10). Perhaps control over microtubule assembly/disassembly may be an important part in the growth regulation of plant cells also.

ACKNOWLEDGEMENTS

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CHITOSOMES AND THE ORIGIN OF CHITIN MICROFIBRILS

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The regeneration of a wall by a protoplast has been one of the most intriguing questions of present and past protoplast symposia. The state of the art has been expertly reviewed by Necas in a preceding chapter.

My present contribution dwells with just one aspect of fungal wall formation, namely microfibril synthesis. Despite some specific advances in this area in the last few years, I must confess, at the outset, that we are still far from understanding the process of wall regeneration by a protoplast or, for that matter, the normal process of cell wall assembly.

Most of our findings on the synthesis of chitin microfibrils have been recently published [1-5] and reviewed [6,7]. This material was largely the result of a collaborative effort with J. Ruiz-Herrera from the National Polytechnic Institute (Mexico), and C. E. Bracker from Purdue University (USA). For the purpose of this symposium, I shall cover pertinent parts of this study in the form of eight questions.

1) Is the intact protoplast needed for microfibril formation? In so far as chitin is concerned, this question can now be answered with a categoric no. The question, however, is not as trivial as it might otherwise appear. Our previous experience, particularly on zoospore encystment of Phytophthora (a case of de novo synthesis of a cellulosic microfibrillar wall by a natural protoplast [8]), had led us to suspect that microfibril formation might require conditions met only by the whole protoplast. We know that zoospores can form a well defined cyst wall in about 2 min [9]. The cells appear to be fully programmed, enzymatically, to make a cell wall and no new protein synthesis is required for encystment. Yet, despite this readiness, if the cell is broken, the ability to form microfibrils disappears and none of it can be recovered in the cell-free extract. All our attempts to produce cellulosic microfibrils in vitro with cell-free extracts of Phytophthora have failed. We have thus encountered what the plant biochemists have also faced--an intriguing inability to reproduce the synthesis of cellulose fibrils in vitro.

But with another fungus--Mucor rouxii, with another kind of cell--budding yeast cell, and with another polymer--chitin, it has been possible to

duplicate the process of microfibril formation in vitro. By a procedure of substrate-induced "solubilization," we purified chitin synthetase and with the resulting transparent "solutions," we showed that chitin microfibrils could be made in vitro [1]. Such microfibrils were indistinguishable from native microfibrils by electron microscopy (shadow casting) and X-ray diffraction.

We conclude that the process of chitin microfibril synthesis does not necessarily require any special condition provided by the intact protoplast*; possibly, the same can be said of other wall microfibrils though no other has been synthesized in vitro.

2) Where is chitin synthetase localized in the cell? Our work on Mucor rouxii yeast cells showed that a major portion of the chitin synthetase of the cell can be recovered as a highly homogenous population (Fig. 1) of minute particles (Fig. 2) in the size range of viruses. These particles were characterized and named chitosomes [3].

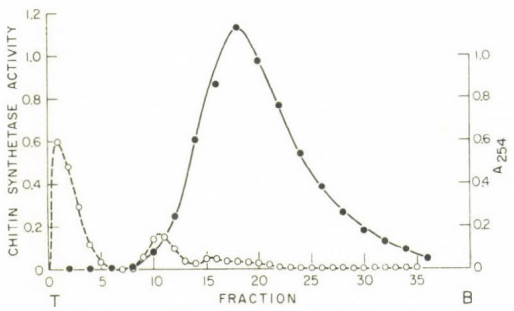

Fig. 1. Sedimentation of chitosomal chitin synthetase in a 5-20% sucrose gradient, centrifuged for 3 h at 81,500 g. Chitin synthetase activity (o). Absorbance (●) [4].

The chitosome isolation procedure involves cell breakage, differential centrifugation, gel filtration, ribonuclease digestion, ultrafiltration and sucrose density gradient centrifugation [3-5]. Routinely, about 20-30% of the initial activity of the cell-free extract can be recovered in the purified pool of chitosomes. However, if procedural losses are minimized, or accounted for, we have estimated that at least 70% of the chitin synthetase of the cell-free extract is in the form of chitosomes. The rest of the activity is associated with the cell wall and with a mixed membrane fraction.

Most chitosomes measure 40-70 nm in diameter and are delimited by a shell which, in thin section, exhibits the double-track appearance of a biological unit membrane (Fig. 3). Whether this is truly a unit membrane remains

* However, it is quite possible that conditions at the protoplast surface may have an important modulating influence on the formation and disposition of microfibrils.

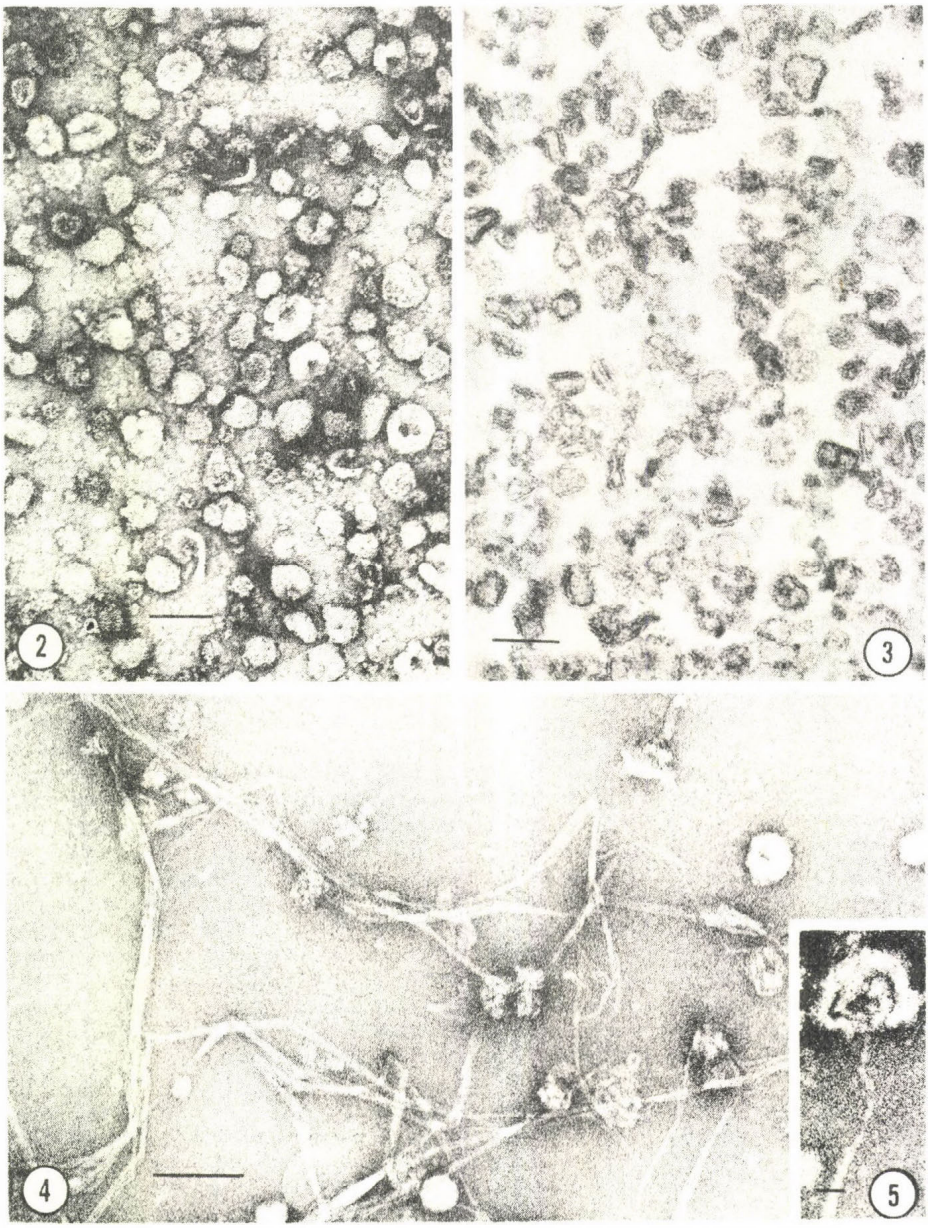

Fig. 2. Electron microscopy of purified chitosomes from yeast cells of Mucor rouxii after negative staining [5].
Fig. 3. Same but in thin section [7].
Fig. 4. Chitin microfibrils synthesized by purified chitosomes from M. rouxii [5].
Fig. 5. Chitosome with a broken shell and extended microfibril emanating from internal fibroid coil [3]. Bar=100 nm (Figs. 2-4) and 20 nm (Fig. 5).

unknown. Chemical analysis of chitosomes made by J. Ruiz-Herrera and coworkers [10], indicated an unusual composition. The ratio of protein/lipid was ~3/1; sterols were the most abundant lipids, and phospholipids were only minor constituents. After activation with proteases and addition of substrate (UDP-GlcNAc) plus GlcNAc (an allosteric activator), chitosomes synthesize chitin microfibrils (Fig. 4). During this process, the chitosome undergoes a profound transformation [3] and the shell or membrane disintegrates but occasionally images can be found of microfibrils arising from a chitosome with a nearly intact shell (Fig. 5). Such images provide strong evidence that chitin microfibrils are made by chitosomes.

In conclusion, our evidence shows that most of the chitin synthetase of actively growing yeast cells of M. rouxii is in the form of chitosomes. These microvesicular organelles are probably the cytoplasmic vehicles that deliver to the sites of wall synthesis on the cell surface, organized assemblages of zymogenic chitin synthetase molecules [3,7]. Presumably each chitosome is responsible for the synthesis of one microfibril [7]. Sections of yeast cells of M. rouxii show microvesicles that resemble chitosomes [3]. These can be seen clearly inside the so-called multivesicular bodies or, with much greater difficulty, free in the cytoplasm among a myriad of ribosomes.

3) Are chitosomes true organelles? The question of whether chitosomes are true cellular structures or artifacts of cell rupture has been openly discussed in our previous communications [3,6,7]. Despite some rather compelling reasons to believe that chitosomes are true organelles, I must admit that final proof for the existence of chitosomes in the living cell is still not available. The main argument against chitosomes being real organelles has been the possibility that they may be artificial fragments of other cell organelles, principally plasma membrane, since the latter has been claimed to be the site of chitin synthetase [11-13]. However, we have established that a) the chitosome membrane does not have, in section, the dimensions or the appearance of plasma membrane [3]; b) chitosomes lack the ATPase activity characteristic of plasma membranes (J. Ruiz-Herrera, personal communication); c) similar chitosome populations, in quality and quantity, result from cells broken by harsh ballistic disruption with glass beads or by gentler lysis of protoplasts (S. Bartnicki-Garcia, C. E. Bracker, and J. Ruiz-Herrera, unpublished); d) upon disruption, the fungal plasma membrane produces vesicles that are of a much larger size range than the chitosome population [e.g., 14]; and e) sections of fungal cells do show microvesicles similar to chitosomes [3,7].

4) What is the smallest chitin synthesizing entity? So far, we have found no evidence for chitin synthesizing particles smaller than chitosomes in cell-free extracts of fungi. The chitosome appears to be the cytological unit of chitin synthesis. It is, however, possible to artificially dissociate chitosomes into much smaller entities or chitosome subunits. Following earlier observations by Gooday and de Rousset-Hall [15] and Duran and Cabib [16] on the "solubilizing" effect of digitonin on crude preparations of chitin synthetase, we were able to dissociate chitosomes with 0.5% digitonin and obtain a highly homogeneous population of particles--chitosome subunits--measuring 7-12 nm diam, with a sedimentation coefficient of 16 S and an estimated molecular weight of 500,000 [17]. Similar particles may be isolated from mycelial wall preparations of M. rouxii (Fig. 6) [18].

The dissociation process is reversible and upon removal or dilution of digitonin by centrifugation through a sucrose density gradient, a partial reassembly of chitosome-like structures can be observed (Fig. 7). The reassembled structures retain the capacity to synthesize chitin microfibrils in vitro. Zymogenicity is preserved during dissociation and reassembly. Optimal conditions and ingredients needed for full chitosome reassembly are yet to be determined. Seemingly, sterols play a crucial role in the structure of chitosomes [10,17].

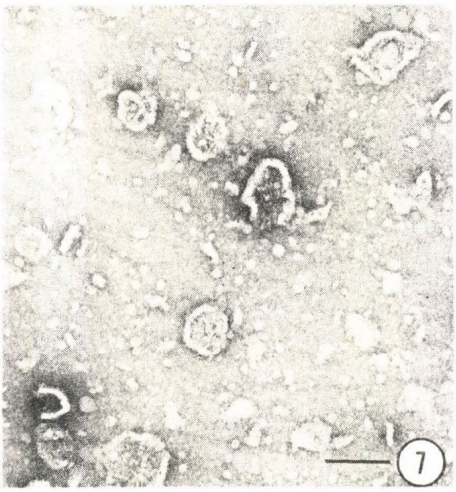

Fig. 6. Purified 16 S subunits isolated by digitonin extraction from mycelial wall preparations of Mucor rouxii.
Fig. 7. Reaggregation of such subunits into vesiculoid, chitosome-like structures, upon removal of digitonin by sucrose density centrifugation.
Bar = 100 nm [18].

In short, chitosomes may be regarded as the cytoloplasmic units of chitin synthetase, and these in turn are made of 16 S subunits with an intrinsic capacity for self-assembly into vesiculoid structures.

5) What regulates the activity of chitosomes? Temporal and spatial controls must operate to regulate the activity of chitosomes in time and space so that microfibrils be produced at the right time and place.

Zymogenicity, a property of chitin synthetase first described by Cabib and Farkas [19], is probably the main device by which chitosomes remain inactive in the cytoplasm. After reaching the cell surface, the zymogen would be activated presumably by a local proteolytic agent. So far, we have no solid indication that chitosomes contain their own activating mechanism and we must therefore invoke an interaction with a surface or periplasmic protease to explain zymogen activation.

We have also isolated a soluble inhibitory protein from the cytoplasm of M. rouxii [20] which is a potent inhibitor of the activity (not activation) of chitin synthetase, but whose role in vivo is not yet defined.

The spatial mechanisms and controls needed to regulate the orderly deployment of chitosomes in the fungal cell are unknown. Our understanding of organelle movement and disposition in the living eucaryotic cell is now rapidly evolving and further discussion of this topic is probably best left for a future occasion.

6) How do chitosomes function in vitro? Where is chitin made by the living cell? At the moment, these are our most important questions, for which unequivocal experimental evidence is not yet available. We have contemplated two major possibilities [7] (Fig. 8):

a) The chitosome becomes integrated with the plasma membrane. In this rather conventional view, the chitosome would fuse with the plasma membrane in the same manner that vesicles are known to do during secretory activity. The fusion process would leave an organized patch of chitin synthetase molecules whose operation would result in the formation of long chitin microfibrils. Evidence supporting localization of chitin synthetase in the plasma membrane has been published [11-13]. Although some of the evidence, particularly that from Cabib's group [12], is highly suggestive, it is not rigorously conclusive; their plasma membrane preparations, despite a high level of purity, are still contaminated by particulate or small vesicular material which might account for all of the chitin synthetase activity.

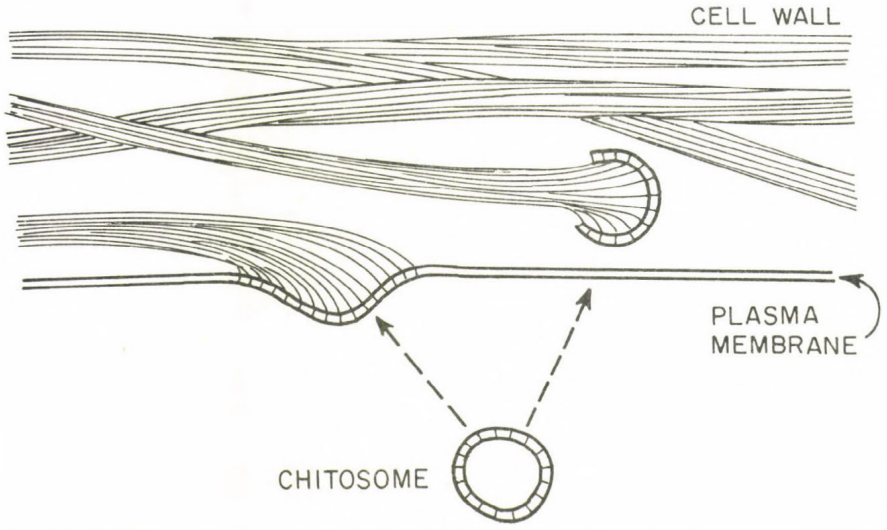

Fig. 8. Two hypothetical mechanisms for the operation of chitosomes in vivo.

b) The chitosome operates largely as an independent structure. Upon reaching its destination somewhere at the plasma membrane/wall interphase,

the chitosome would synthesize microfibrils in a manner analogous to that observed in vitro (Figs. 5, 8). There is no experimental proof for this view, but the chitin synthetase activity found tightly associated to cell walls of M. rouxii [21] may correspond to chitosomes positioned at their final "periplasmic" destination.

7) Are chitosomes present in other fungi? Chitosomes have been isolated from a variety of fungi belonging to major taxonomic groups [5]. In all cases examined, a major portion of the total chitin synthetase activity of the cell-free extracts was recovered in the form of particles that exhibited the same sedimentability, morphology, and size range, as the chitosomes isolated from yeast cells of M. rouxii. The specimens include the mycelial phase of Mucor rouxii, the mycelium of an aquatic mold (Allomyces macrogynus), budding cells of the hemiascomycetous yeast (Saccharomyces cerevisiae), the mycelium of an ascomycete (Neurospora crassa), and the basidiocarp of a common mushroom (Agaricus bisporus). Fig. 9 illustrates the chitosomes isolated from S. cerevisiae. These preparations were similar to those of M. rouxii, but differed in two respects; first, it was quite difficult to eliminate ribosomes from the S. cerevisiae samples; second, the zymogen of S. cerevisiae responded poorly to acid proteases but well to neutral proteases, whereas the reverse was true for the zymogen of M. rouxii [5]. The chitosomes from Saccharomyces cerevisiae produced long microfibrils (Fig. 10) similar to those obtained from M. rouxii.

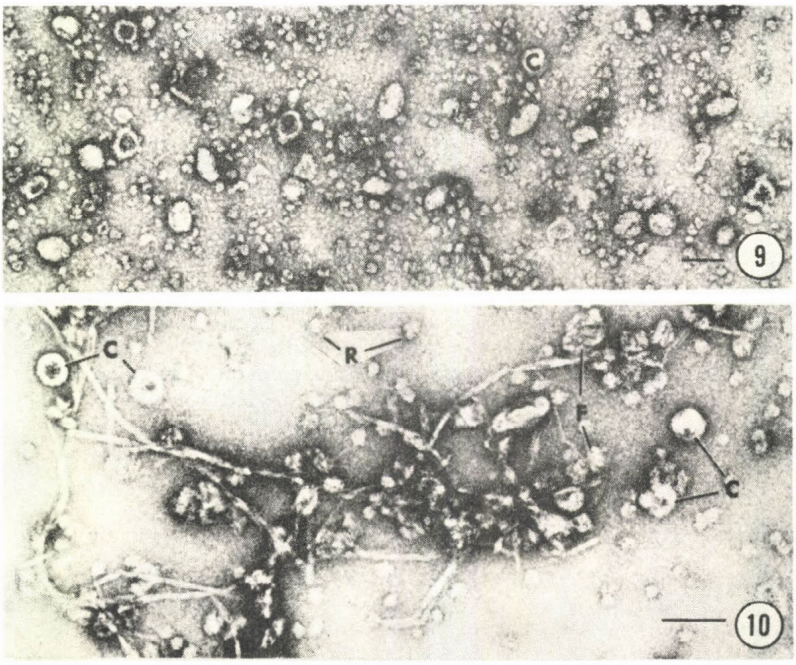

Fig. 9. Purified chitosome preparation from Saccharomyces cerevisiae. Note abundance of contaminating ribosomes that survived RNase treatment. Bar = 100 nm.

Fig. 10. Chitin microfibrils synthesized by chitosomes from S. cerevisiae [5]. C, chitosomes; R, ribosomes; F, fibroid coils. Bar = 100 nm.

On the basis of our admittedly small but diverse sample of fungi, we have proposed that the organization of chitin synthetase into chitosomes is an ubiquitous feature of all chitinous fungi [5].

8) How is the synthesis of a chitin chain initiated? Since the discovery of chitin synthetase, the question of chitin-chain initiation has been pondered. Various primers or initiators have been proposed: chitodextrins [22], GlcNAc [23], or a glycolipid [24]. In recent years, proteins have been shown or proposed to serve as primers or acceptors for the synthesis of polysaccharides, [25]. In view of the availability of a highly purified system of chitin synthetase, i.e., chitosomes, we decided to search for evidence that chitin chains are linked to protein.

Chemical analysis of microfibrils synthesized in vitro by either chitosomes from yeast cells of Mucor rouxii or digitonin-dissociated enzyme (from mycelial walls of M. rouxii) contained a small but significant proportion of amino acids after 6N HCl hydrolysis [26]. This proteinaceous material was firmly bound and was only partly released by harsh extraction treatments. Likewise, chitin isolated from cell walls of M. rouxii has polypeptide material firmly bound to it [26]. We have proposed that this polypeptide served as the acceptor for the initiation of chitin chains. There is evidence that exogenously supplied polypeptides, e.g., poly-L-lysine, may compete with the presumed acceptor of chitin synthesis.

Tunicamycin [27], an inhibitor of protein glycosylation (via dolichol intermediates [28]), was found to inhibit chitin synthetase of M. rouxii [26].

We have tentatively suggested that chitin-chain synthesis is a two-stage process: a chain initiation stage via glycosylation of a polypeptide acceptor and a subsequent stage of chain elongation by repeated transfer of GlcNAc from UDP-GlcNAc.

CONCLUSION

The chitosome appears to be a central piece in the cellular machinery of fungal wall synthesis; its mission is to deliver an organized package of chitin synthetase zymogen to specific sites where cell wall growth takes place. The chitosome is made of 16 S subunits. Conceivably, the concerted operation of the organized subunits ensures the orderly formation of long, regular microfibrils.

ACKNOWLEDGMENT

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POLYAMINES, MACROMOLECULAR SYNTHESES AND THE PROBLEM OF CEREAL PROTOPLAST REGENERATION

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INTRODUCTION

Protoplasts obtained from leaves of certain plants, especially members of the Solanaceae, can synthesize new walls, undergo nuclear and cellular division and ultimately regenerate entire plants (1,2). By contrast, protoplasts from leaves of cereals do not undergo sustained mitotic division when cultured under similar conditions on a wide range of media (3,4). Non-dividing cereal protoplasts manifest a progressive decrease in rate of incorporation of amino acids and nucleosides into presumptive protein and nucleic acids, respectively (5), as well as an increase in potentially detrimental hydrolases such as RNase and protease (6). This decrease in net synthetic activity and lack of mitosis in cereal protoplasts may result from a complex of senescence induced changes (7) or injury caused by plasmolysis (8) that must precede protoplast isolation. These stresses can produce severe metabolic alterations, contributing to a blockage of the cell cycle.

Recently, substantial evidence has accumulated suggesting an important role of polyamines in regulating DNA synthesis and an orderly progression through the animal cell cycle; similar evidence is far less extensive for plants. Accordingly, we have investigated the possible importance of polyamines in facilitating cereal protoplast division.

Polyamines

Polyamines such as putrescine, cadaverine, spermidine and spermine, which occur widely in prokaryotes and eukaryotes, have been known for over 300 years, but their physiological importance has only recently been investigated. There is now much evidence that they play a significant role in the biosynthesis and function of nucleic acids in various biological systems (9,10), and it is probably through their interaction with these macromolecules that the polyamines are able to promote growth in certain microorganisms and animal cells. By contrast, comparatively little is known about the biological significance of polyamines in plants. In view of our recent experiments indicating their probable importance in physiological regulation in higher plants, we shall review briefly their occurrence and biochemical role, giving special emphasis to their possible effects on the control of physiological functions in plants.

Crystals of spermine phosphate were observed by van Leeuwenhoek (11) in

human semen; other polyamines are generally found in abundance in cells whose nuclei are very active. Polyamines are probably ubiquitous in biological materials, although the relative amounts of putrescine, spermidine and spermine differ greatly in different cells. Prokaryotes have a higher concentration of putrescine than spermidine and lack spermine, while eukaryotes usually have only trace amounts of putrescine and higher concentrations of spermine and spermidine.

In plants, most research on polyamines has been centered on putrescine, which has been reported in algae (12), fungi (9) and several species of higher plants (9,13). Spermidine has been identified in Chlorella (12), leaves of tomato and Chinese cabbage (14) and together with spermine, in pollen grains of Petunia (15). High concentrations of spermidine and spermine have been reported in the embryo (16) and in several other parts of cereal plants (13,17), as well as in tubers of Jerusalem artichoke (18) and in seeds from a large variety of higher plants (19). Spermidine and spermine also occur as conjugates of a number of plant alkaloids (13). Recently all of the above polyamines were detected in a number of leguminous plants (20,21), while cadaverine has been identified only sporadically in cereals and other families of higher plants (22).

There are reports suggesting that a substantial proportion of the intracellular polyamines is attached to ribosomes and that spermine, but not spermidine, is concentrated in the nucleus (23,24). In plants, the distribution of spermidine and spermine in both the subcellular particles as well as in the soluble fraction of etiolated epicotyls of Pisum sativum has been investigated (25). In chloroplasts of Euglena and spinach leaves, the ratios between spermidine/spermine and putrescine/spermine are lower than in the complete cell (25).

The pathway for the biosynthesis of putrescine and spermidine was first established in microorganisms and was later found to be very similar in animal cells (26). Putrescine arises from arginine by one of two pathways: Arginine can lose urea to become ornithine, and the latter can be decarboxylated by the enzyme ornithine decarboxylase (ODC) to form putrescine. Alternatively, arginine can be decarboxylated by the enzyme arginine decarboxylase (ADC) to yield agmatine; the latter can then lose urea to become putrescine. The second pathway seems to occur preferentially in plants (9, 27). Spermidine and spermine are then formed from putrescine by the consecutive addition of propylamino residues donated by decarboxylated S-adenosylmethionine (SAM) generated via S-adenosylmethionine decarboxylase (SAMD) (9,14). By a separate pathway, cadaverine may be formed from decarboxylation of lysine (9,13).

The polyamines are catabolized by various widely distributed amine oxidases which probably serve to regulate intracellular polyamine levels. These enzymes function by a variety of mechanisms: In bacteria, the bonds adjacent to the secondary amine group of the polyamines are attacked, splitting the molecule to form 1, 4-diaminobutane (putrescine) or 1, 3-diaminopropane. In animals, plasma amine oxidase oxidizes the primary amino groups of tri- and tetra-amines only, giving rise to dialdehydes (28).

In higher plants, especially in legumes, diamine oxidase has been reported to catalyze the oxidation of primary amine groups of both the diamines and polyamines (13,29). Diamine oxidase activity was not detected in cotyledons of ungerminated pea seeds but increased significantly within

a few hours after germination. Soybean diamine oxidase resembles the enzyme from pea, but does not appear in the cotyledons until at least 6 days after germination, and reaches maximum activity in roots and hypocotyls 1 and 3 days respectively after germination (13). The polyamine oxidase which oxidizes spermidine and spermine has been found in the leaves of many Gramineae but not in the leaves of many other mono- or dicotyledonous plants investigated (30). This enzyme is most active on spermine, oxidizing it to 1, 3-diaminopropane and 1(3-aminopropyl)-pyrroline. In oats, the polyamine oxidase activity is high in seedlings grown in the dark and low in seedlings grown in the light (13).

Polyamines behave as cations at physiological pH and are known to bind strongly to nucleic acids and proteins containing negatively charged groups. They associate with DNA and RNA, and are known to stabilize the secondary structure of these compounds, thereby making them more resistant to nuclease and thermal denaturation. In cell-free systems from bacterial and mammalian cells, every step in polypeptide synthesis can be stimulated by addition of putrescine, spermidine or spermine (9,10,14). In polyamine-deficient mutants of E. coli, polyamines have been reported to participate directly in protein synthesis (31); for plants, the limited evidence suggests an analogous relationship between these amines and macromolecular metabolism (32,33,13).

Effects of polyamines have been noted on RNA polymerase, DNA polymerase and ribonuclease. In bacterial and mammalian systems, polyamines at low concentrations enhance, while high concentrations inhibit DNA-dependent RNA polymerase (9,24). In plants also, spermidine increased the activity of RNA polymerase in preparations from maize seedlings (34), Helianthus tuberosus (19), and soybean hypocotyl (35). In contrast, the effects of polyamines on DNA polymerase are not clearly understood, although it has been suggested that the amines interact with the nucleic acids rather than with the enzymes (24).

Spermidine and spermine have been reported to inhibit the activity of purified ribonuclease preparations in several other systems (24,36,37). Several groups of workers(38) suggested that spermine inhibits RNase activity by binding to phosphate groups of RNA, thereby protecting the internucleotide linkages from degradation. More recently spermidine and spermine have been reported to promote, rather than inhibit, RNase activity in bacterial and mammalian cells. However, these RNases have specificity for bonds involving pyrimidines rather than purines (39, 40).

Putrescine and related amines have been described as growth factors in some microorganisms, mammalian cell lines and higher plants (9,10,26,41). Spermidine concentration is highest in newborn rat tissues and decreases steadily with age (42). If the rats are injected with sufficient growth hormone to induce an increase in RNA synthesis, there is a rise in incorporation of aminopropyl fragments from methionine into spermidine. Inhibition of spermidine and spermine synthesis in rat embryo fibroblasts by methylglyoxal-bis(guanylhydrazone) (MGBG), which is known to block SAMD activity, results in growth arrest in the G1 phase of the cell cycle. Addition to spermidine or spermine to such MGBG-inhibited cells results in rapid resumption of proliferation (43). Similar correlations between growth and polyamine biosynthesis have been shown in Chinese hamster ovary cells (44) and in rat liver tumors (45).

While very little is known about the biochemical role of polyamines in plants, increasing attention is now being given to their role as growth factors. These amines are present in trace amounts in dormant tubers of Helianthus tuberosus but increase 10-20 fold when the tubers start to grow. In vitro, growth of the tuber slices is stimulated by polyamines and this stimulation is similar to that observed by IAA treatment. Since IAA application results in polyamine accumulation, it was proposed that a rise in polyamine titer might mediate some of the growth effects of IAA (18). Similar increases in spermidine accumulation were obtained after auxin (NAA) treatment (46). As in animal tissues, plant tumors contain higher levels of polyamines than do normal cells; spermine levels rise threefold, while putrescine levels can rise 100 fold or more after tumor induction (47). Putrescine levels are also known to rise remarkably in several widely differing species of higher plants when they are grown in potassium or magnesium-deficient conditions (13). Increased levels of putrescine were also reported in broad-bean leaves when the plants were grown in NaCl-rich medium (9). Infiltration of cadaverine or putrescine into developing ears of wheat increased nucleic acid content and growth of seeds (48).

Variations of polyamine concentrations during seedling growth may be related to their function as growth factors in some plants. For example, during the growth of Phaseolus vulgaris seedlings, spermidine and spermine decrease in cotyledons and increase in shoots, with simultaneous increases in levels of RNA and proteins and growth (49). More recently, a close relationship was shown to exist between the site and magnitude of polyamine accumulation and the contents of nucleic acids and proteins in the rapidly growing embryo-axis of Lathyrus sativus (50). Accumulation of cadaverine was associated with rise in nucleic acid and protein contents during embryonic development and growth. A similar correlation between polyamine concentration and RNA, but not DNA, content was observed in suspension cultures of Paul's Scarlet rose cells (51). Polyamines have also been shown to affect the activity or level of numerous enzymes in plants (13). Most recently, it has been suggested that these polyamines may play a role in cellular differentiation during embryogenesis of carrot cell cultures, since putrescine and spermidine levels (52) as well as arginine decarboxylase activity (53) increase within 24 hrs after transfer of cells to embryogenic medium.

MATERIALS AND METHODS

Protoplast isolation

The first leaf of 7-day-old seedlings of Avena sativa L. (var. Victory) was used for isolation of protoplasts. The seeds were grown in vermiculite in controlled growth rooms with a 16-hr photoperiod of 12,000 lux as detailed in an earlier report (5).

The leaves were sterilized by immersion in 70% ethanol for 2 minutes, 2 washes with sterile distilled water, further dipping in 10% Clorox for 5 minutes containing Tween 20 (1 drop/10 ml), and finally 5-6 rinses with sterile distilled water. All manipulations were performed aseptically in a laminar flow hood.

Protoplasts were isolated by stripping off the lower epidermis and floating the leaves, stripped side down, on 0.5% w/v Cellulysin (B grade, Calbiochem) in B5 medium (3) or in 1 mM phosphate buffer, pH 5.8, with

0.4 M sorbitol or 0.6 M mannitol for 2 hr at 30 ± 1 C. The released protoplasts were collected by centrifugation at 50 g for 5 min, then washed 3 times with the above medium by centrifugation and resuspension. The final protoplast pellet was resuspended at a concentration of ca 3 x 10^5 protoplasts/ml and cultured in B5 medium with the following additives: 100 g/l mannitol, 15 g/l sucrose, 2 mg/l α-naphthylacetic acid (NAA), 1 mg/l benzyladenine (BA), 6000 mg/l $CaCl_2$, 100 mg/l L-ascorbate, 100 mg/l inositol, 250 mg/l xylose, 250 mg/l arabinose, and 1 mM of any of the three polyamines (cadaverine, spermine, spermidine) as their HCl salts (Sigma). The culture medium was filter-sterilized and the pH was adjusted to 5.8. Control treatments included no polyamines. The protoplast suspensions were cultured as 20 λ drops hanging from covers of Petri dishes; 5 ml of the same medium was included in the bottom of each Petri dish to establish vapor phase equilibrium with the drops. The dishes were placed in moist chambers in the dark and the protoplasts were cultured for varying lengths of time at room temperature (ca 23 C).

Labeling experiments

Aliquots of one half ml suspensions of protoplasts containing 2-5 x 10^5 protoplasts/ml were incubated usually for 4 hr with 20 µl of 10 µ Ci/ml L-leucine ([4,5-^{3}H(N)], specific radioactivity 60 Ci/m mole); 100 µ Ci/ml uridine ([5-^{3}H], specific radioactivity 27.6 Ci/m mole); or 100 µ Ci/ml thymidine (methyl-^{3}H), specific radioactivity 56.4 Ci/m mole). All radioactive precursors were purchased from New England Nuclear Co. The incubation was performed in triplicate in covered disposable microbeakers in a Dubnoff metabolic shaking incubator (40 reciprocal strokes/min) at 23 ± 1 C. At the end of each incubation period, 20-50 µl aliquots were pipetted onto discs of Whatman No. 3 MM filter paper and the incorporation of label into TCA-insoluble materials was measured as described in an earlier paper (5). The washed and air-dried discs were placed in 2 ml of Aquasol (New England Nuclear) in minivials, and radioactivity determined in an Ansitron scintillation counter.

The specificity of incorporation of each precursor was assessed by determination of the degree to which the incorporation product could be hydrolyzed by appropriate specific enzymes. Leucine incorporation into protein was tested by pronase (Calbiochem) incubated at 37 C for 1 hr prior to use to inactivate DNase and RNase. Filter paper discs containing the labelled material were incubated at 37 C for 1 hr in 50 µg/ml pronase in 100 mM TRIS buffer, pH 8.0. Uridine incorporation into RNA was tested by pancreatic RNase (Worthington), heated 5 min at 100 C prior to use to eliminate DNase and protease activity. Discs were incubated in 50 µg/ml RNase in 100 mM acetate buffer pH 5.5. Thymidine incorporation into DNA was tested by DNase (Worthington) stipulated as being RNase-free. Discs were incubated in 50 µg/ml DNase in 25 mM TRIS buffer, pH 7, with 5 mM $MgCl_2$. The activity of this DNase was tested against ^{3}H-labelled λ-phage DNA. Over 90% of the counts from this material was solubilized by the enzyme. Preliminary experiments revealed that no counts from labelled macromolecules were lost from the filter paper discs during incubation in buffer alone; only active enzyme appeared to solubilize, and thus remove the counts from each of the labelled precursors. In each case, after appropriate enzymatic treatment, the residual undigested macromolecule on the filter paper was precipitated with trichloroacetic acid, processed as described above and counted.

Staining procedures

Mitotic activity was determined by direct observation, by counting nuclei, and by staining the protoplasts with 1% aceto-carmine or modified carbolfuchsin. Briefly, aliquots of the protoplast suspension were mixed with the fixative acetic acid:ethanol in a 1:9 ratio (v/v) in 0.2 M mannitol and kept at 4 C for 24 hr. The fixed protoplasts were stained and then gently spread to visualize the chromosomes without excessive crushing of the cell. The data presented are from single experiments, which generally were representative of a number of experiments (2-4) in each treatment.

RESULTS AND DISCUSSION

Characterization of macromolecular products.

Freshly isolated protoplasts from oat leaves, despite the injury and osmotic stress experienced during isolation, are able to incorporate ^{3}H-leucine, uridine and thymidine into trichloroacetic acid-insoluble macromolecules (Table I; see also reference 5). The net incorporation increased with time in culture for 18 hr. Separate digestion of the trichloroacetic acid-insoluble components with pronase and RNase showed that in both fresh and 18-hr old protoplasts, over 70% of the leucine and uridine were incorporated into authentic protein and RNA, respectively. In contrast, none of the thymidine was incorporated into DNA of freshly isolated protoplasts, and only about 13% was incorporated into DNA of the cultures aged for 18 hrs before exposure to ^{3}H-thymidine (Table 1). Even when the protoplasts were cultured for longer periods (4-8 days), DNA synthesis did not occur significantly and the protoplasts did not show any mitotic division. In tobacco protoplasts, on the other hand, both incorporation of thymidine into DNA and cell division have been observed (54). Thus, oat protoplasts can synthesize abundant protein and RNA, but not significant quantities of DNA, and hence appear to be arrested at the G_1 phase of the cell cycle.

Effect of polyamines on incorporation of thymidine.

The ability of oat protoplasts to incorporate ^{3}H-thymidine into trichloroacetic acid-insoluble material decreases rapidly with time of culture of protoplasts after 18 hr (Table 2). Treatments with cadaverine, spermidine or spermine not only prevent this decline, but increase the extent of incorporation of thymidine into trichloroacetic acid-insoluble materials. Spermine treatment were most effective in enhancing incorporation and in stabilizing the protoplasts during culture.

The rapid decline in the ability of the control protoplasts to synthesize DNA during culture suggests that these protoplasts are subject to postisolation senescence. Reduction of such senescence, as shown by polyamine-induced net increase in thymidine incorporation, is supported by earlier observations of polyamines as senescence inhibitors in oat leaves (55), oat protoplasts (6,7,56), and certain mammalian cells (8).

Effect of polyamines on DNA synthesis and mitotic activity.

Measurements of the amount of incorporated ^{3}H-thymidine removed by digestion with DNase (Table 3) show that only about 12% of such thymidine was present in DNA in control protoplasts. Addition of cadaverine or

spermine to the culture medium increased this value to about 18% and 21%, respectively. Although these polyamine-mediated increases in DNA synthesis are relatively small, they are consistent and significant, not only in the 4-day old cultures but also in cultures continued for longer periods.

The increased DNA synthesis led us to investigate the effect of polyamines on mitotic activity. While control protoplasts showed only sporadic nuclear divisions, treatments with 1 mM spermidine or spermine significantly increased their frequency (Table 4). Polyamine treatments produced numerous binucleate cells as well as typical mitotic figures (Figure 1). These observations demonstrate that (a) oat leaf protoplasts cultured on usual defined media are able to synthesize abundant proteins and RNA but not substantial quantities of DNA, and (b) treatments with polyamines measurably increase DNA synthesis as well as mitotic activity in these cells.

The implications of this finding for future work on cereal protoplast culture may be profound. It is important to recognize, however, that the permeability of the protoplast to polyamines is limited, and that polyamine oxidase may destroy those polyamines that penetrate. Much more research is needed before this can become a standard method. Most promising at present is the combine use of polyamines, pinocytosis inducers and polyamine oxidase inhibitors.

SUMMARY

Polyamines, known to increase nucleic acid synthesis and mitosis in various animal and microbial systems, have been applied to the problem of cereal protoplast culture. Freshly isolated protoplasts from leaves of oat seedlings (Avena sativa L. var. Victory) do not divide when cultured on a wide range of media, but are capable of incorporating tritiated leucine, uridine and thymidine into trichloroacetic acid-insoluble macromolecules. Over 70% of the leucine and uridine incorporated over an 18-hr period are found in protein and RNA, respectively, as shown by hydrolysis of the macromolecular products with a specific protease or RNase. However, little or none of the tritiated thymidine is incorporated into macromolecules hydrolyzable by DNase over an 18-96 hr period, and even this incorporation of thymidine into trichloroacetic acid-insoluble material declines sharply with increasing time of culture after 18 hr. Addition of di- or polyamines such as putrescine, cadaverine, spermidine and spermine to the medium not only prevents the decline, but actually increases net incorporation of all precursors, including a fraction of thymidine going into DNA. A significant increase in mitoses and binucleate protoplasts is also observed in 72-168 hr cultures.

The inability of freshly isolated oat leaf protoplasts to synthesize significant quantities of DNA suggests that they are arrested at the G_1 phase of the cell cycle. Treatment with polyamines appears to promote both DNA synthesis and the inception of mitotic activity in oat protoplasts, as in numerous animal and microbial cells.

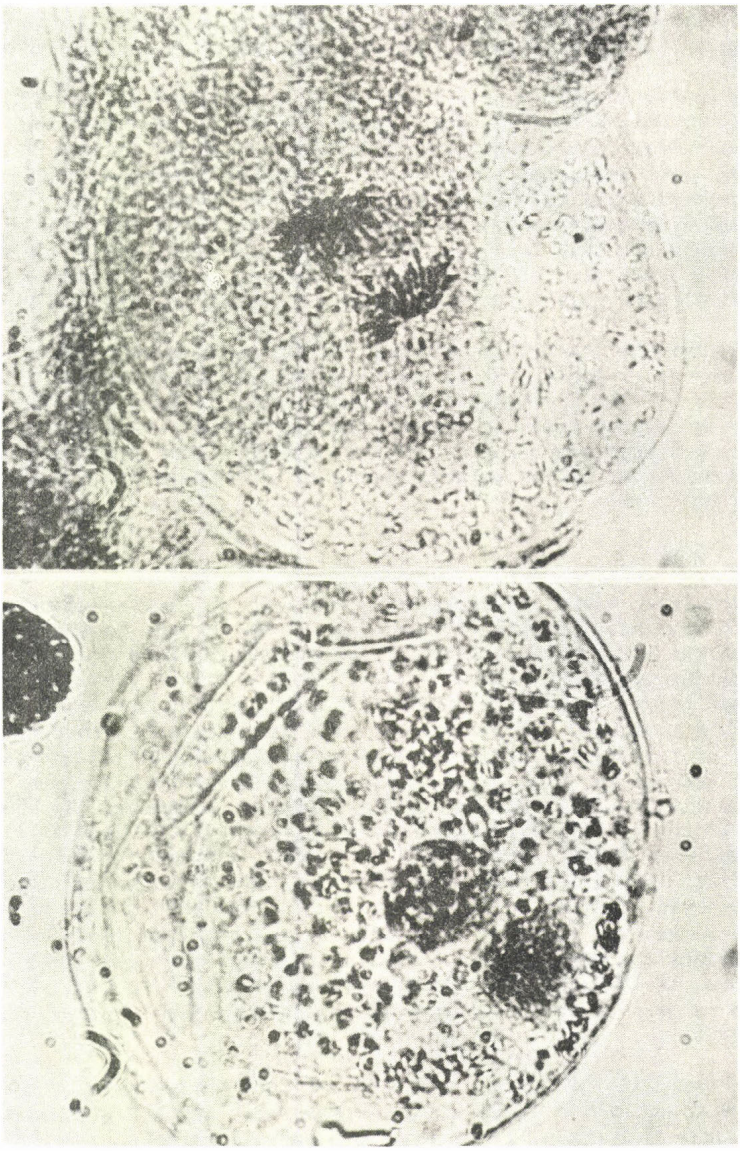

Figure 1 Mitosis in oat leaf protoplasts

A/ Anaphase from 120 hr culture in B5 medium + 1 mM spermidine /400 x/ and B/, binucleate cell from 120 hr cultures in B5 medium + 1 mM spermidine /400 x/. Protoplast cultures were fixed in 1:3 acetic acid/ethanol and stained with 1 % aceto-carmine

TABLE 1 Characterization of macromolecular products resulting from incorporation of tritium labelled leucine, uridine and thymidine into oat leaf protoplasts

Age of protoplasts, hr	CPM/5 x 10^4 protoplasts								
	^{3}H-leucine			^{3}H-uridine			^{3}H-thymidine		
	Pronase -	Pronase +	% incorporation into protein	RNase -	RNase +	% incorporation into RNA	DNase -	DNase +	% incorporation into DNA
0	514	120	77	310	77	75	173	171	0
18	4852	836	83	1301	376	71	284	247	13

TABLE 2 Effect of polyamines on incorporation of ^{3}H-thymidine into TCA-insoluble material by oat leaf protoplasts cultured as hanging drops

Incubation medium	^{3}H-thymidine incorporation			
	18 hr		96 hr	
	absolute cpm/6 x 10^3 protoplasts	relative /%/	absolute cpm/6 x 10^3 protoplasts	relative /%/
Control, B5 medium	284	100	171	100
+ Cadaverine, 1 mM	355	125	298	174
+ Spermidine, 1 mM	433	152	385	224
+ Spermine, 1 mM	413	145	412	240

TABLE 3 Effect of polyamines on incorporation of ^{3}H-thymidine into DNA of oat leaf protoplasts cultured as hanging drops for 96 hr

Incubation medium	^{3}H-tymidine, cpm/6 x 10^{3} protoplasts			% Incorporation into DNA
	-DNase	+DNase	Difference	
Control, B5 medium	284	251	33	12
+ Cadaverine, 1 mM	326	267	59	18
+ Spermine, 1 mM	412	328	84	21

TABLE 4 Effect of spermidine on the frequency of binucleate oat leaf protoplasts cultured as hanging drops

Age of cultures in B5 medium, hr	Nuclear counts		% Binucleate protoplasts
	Total protoplasts observed	Binucleate protoplasts	
0	972	2	0.20
18	895	2	0.22
120, control	610	3	-.40
120, 1 mM spermidine	532	12	2.20

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